THE CONTRIBUTION OF STEROIDS TO THE DIGOXIN-LIKE IMMUNOREACTIVE SUBSTANCE IN CORD BLOOD

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Abbreviations :-

ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
17-OHP	17α-hydroxyprogesterone
CV	Coefficient of variation
DHEAS	Dehydroepiandrosterone sulphate
DLIS	Digoxin-like immunoreactive substance
EIA	Enzyme immunoassay
FIA	Fluorescence immunoassay
RIA	Radioimmunoassay
SD	Standard deviation

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1. ABSTRACT

Digoxin-like immunoreactivity has been reported in plasma of neonates, patients with renal failure, liver failure and pregnancy. Thus the assay of digoxin level in these groups may not accurately reflect the true therapeutic level. The cross reactivity can be due to either the non-specificity of the antibody used in the assay or the existence of endogenous compound(s) with similar structure to digoxin. In addition, digoxin-like immunoreactive substance (DLIS) has also been implicated as potential endogenous inhibitor of $[Na^+, K^+]$-ATPase.

Newborn infants and pregnant women are known to have high level of steroid hormones and their intermediates in circulation. Because of structural similarities between steroid hormones and digoxin, the correlation of steroid concentrations and digoxin-like immunoreactivity in cord blood was examined. Cross-reactivity of progesterone, oestradiol, dehydro-epiandrosterone sulphate (DHEAS), androstenedione, 17α-hydroxyprogesterone (17-OHP) and oestriol was studied using a commercial digoxin assay.

It was demonstrated that there is high digoxin-like immunoreactivity in cord blood samples from newborns without previous maternal or foetal digoxin treatment. DLIS varied from 0.50 nmol/l to 1.37 nmol/l digoxin equivalent and the mean was 0.91 nmol/l. When cord blood was heated (5 min at 100° C), the digoxin-like immunoreactivity increased by a mean of 2.3 times. This suggests that DLIS is present in serum in the form of free and bound fractions. The bound fraction is readily dissociated by heating.

Significant correlation between digoxin-like immunoreactivity and the combined effect of steroid hormones was found. It was found that about 68% of the observed digoxin-like immunoreactivity could be explained by the presence of high concentration of steroid hormones in cord blood.

Progesterone was found to have the highest cross-reactivity of 0.0067%. The potency of cross-reactivity in the RIA kit was found to be in the order of progesterone, androstenedione, 17-OHP, cortisol, DHEAS, oestradiol and oestriol. Oestradiol and oestriol had the least cross-reactivity of <0.0002%.

Cord blood samples were also assayed for their ATPase inhibitory levels. A mean value of 26.1

nmol/l ouabain equivalent was found. The ATPase inhibitory properties of the steroids were also studied. In the assay for [Na^+,K^+]-ATPase inhibition study, progesterone was found to be the strongest inhibitor followed by androstenedione, DHEAS, cortisol, oestradiol and oestriol. 17-OHP did not have ATPase inhibitory action even at millimolar concentration.

Overall there was lack of correlation between the biochemical and immunological properties of the steroids as DLIS. Although progesterone showed the highest cross reactivity with the digoxin antibody and, ATPase inhibitory activity, however, its contribution to the digoxin-like immunoreactivity in cord blood was found to be 2-14%.

Although steroids were demonstrated to cross-react with digoxin antibody and inhibit ATPase activity to some degree, their concentrations in plasma cannot totally account for concentration of DLIS in cord blood. Simulated serum cocktails failed to give a comparable digoxin cross-reactivity compared to the corresponding cord blood with similar steroid concentrations. They also failed to inhibit ATPase activity. Preliminary evidence suggests that DLIS immunoreactivity may not be due to the elevation of major known steroids in cord blood.

2. REVIEW

2.1 Chemical nature of digoxin

Digoxin is one of the digitalis compounds (cardiac glycosides) which have a characteristic ring structure (aglycone or genin) to which is coupled one or more sugars. The aglycone portion of the glycoside consists of a steroid nucleus and an lactone ring at C17 position of the steroid nucleus. The hydroxyl group at C3 and C14 are in β-configuration. The sugars are attached usually through the C3 hydroxyl group. Digoxin has three sugar residues attached at C3 position. (Figures 1a and 1b)

2.2 Use of Digoxin

Digoxin can influence the contractility of myocardial muscular tissue (inotropic action) and affect the rate of rhythmic movement such as heart beat (chronotropic action). It is commonly used in the treatment of cardiac failure. It enhances the contractility of myocardial tissue so as to improve the pumping function of the heart. It is also used as an anti-arrhythmic agent for atrial fibrillation and flutter. The mechanism of action of digoxin is through

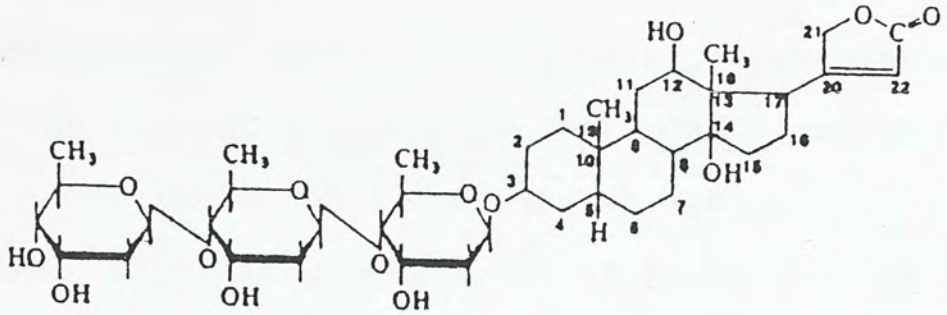
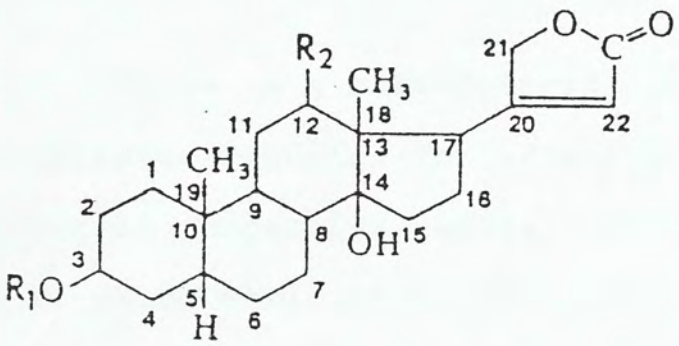


Figure 1a. Chemical structure of digoxin



Compound	R_2	R_1 Sugars at 3 position
Digoxin	OH	tridigitoxose
Digitoxin	H	tridigitoxose
Deslanoside	OH	tridigitoxose - glucose
Lanatoside C	OH	didigitoxose - acetyldigitoxose glucose

Ouabain: has only 1 sugar attached to C3. viz. 6-deoxy-α-mannose. The C19 position contains the $-CH_2OH$ radical instead of $-CH_3$. While a-OH group is on C1, C5, and C11.

Figure 1b. Chemical structure of key cardiac glycosides

its inhibitory action on the [Na^+,K^+]-adenosine triphosphatase (ATPase) (EC 3.6.1.37) transmembrane pump of the sarcolemma [1]. The inhibition results in the accumulation of intracellular sodium ions, which is thought to cause displacement of bound calcium ions. The resulting high calcium ions available to contractile elements cause a positive inotropic effect, resulting in more forceful contraction of the myocardium [2].

2.3 Therapeutic monitoring of digoxin

There is a marked variation in the individual sensitivity to digoxin. The effective dosage of digoxin treatment in congestive cardiac failure and the plasma level at which toxicity occurs among individuals may be different [3]. Moreover, there is substantial overlap between the currently defined therapeutic range and toxic concentration. Therefore, appropriate therapeutic goals should be chosen with care when digoxin is used. Many immunoassays have been developed and marketed for the measurement of digoxin level in patients in order to monitor the plasma level and to detect toxic plasma concentration (above 3.2 nmol/l).

2.4 Digoxin measurements

Historically digoxin assays are of two types: those using [Na^+,K^+]-ATPase inhibition and those using immunoassays with antibodies raised against digoxin.

2.4.1 ATPase-based assays

Since digoxin is known to inhibit ATPase activity, digoxin can be measured by i) the measurment of ouabain sensitive ATPase inhibition, ii) competition for digitalis receptor [4] and iii) the rate of cation transport [5]. Using cells and tissue, one can measure cation transport by monitoring either ^{86}rubidium [^{86}Ru] influx or ^{22}sodium [^{22}Na] efflux. These non-specific methods for the quantitation of digoxin were not explored further and eventually abandoned.

2.4.2 Immunoassays

With the development and introduction of the immunoassays, digoxin measurement by radioimmunoassay (RIA), enzyme immunoassay (EIA) and fluorescence immunoassay (FIA) have become popular. There are several commercial and non-commercial digoxin immunoassays available which are claimed to be relatively specific [6]. The digoxin concentrations measured in plasma or serum usually yield varying results with different immunoassays [7]. Cross-

reactivity and interference with structurally related steroids and other cardioglycosides are stated for most commercial antisera (eg. Table 1 shows cross-reactivities for one of the commercial (RIANEN) digoxin kit). Antibodies used in these assays are raised against a conjugate of bovine albumin and digoxin, which is prepared by coupling linkage through the carbohydrate moiety of digoxin. The antibodies generated against this conjugate are directed against the steroid moiety of digoxin [8] and therefore may cross-react with other related steroids.

2.4.3 Known substances interfering in ATPase assay and digoxin RIA

It was demonstrated that endogenous lipids and fatty acids could inhibit ATPase activity [9,10]. Unsaturated fatty acids [11] and lysophospholipids [10] had been reported to be immunoreactive with anti-digoxin antibodies and also inhibited [Na^+,K^+]-ATPase. It was demonstrated by Lau and Valdes that in the presence of protein, the ATPase inhibitory activity and interaction with the anti-digoxin antibody were virtually eliminated [12].

Moreover, [Na^+,K^+]-ATPase is very sensitive to intracellular sodium, and therefore substances including thyroxine [13] and aldosterone [14] that

Table 1 Cross reactivity at 50% displacement compared to digoxin :-

Digoxin	100%
Deslanoside	70.4%
Lanatoside	38.9%
Digitoxin	5.3%
Acetyldigitoxin	1.1%
Progesterone	0.056%
Ouabain, Prednisolone, Cortisol	<0.02%
Testosterone, corticosterone, Deoxycortisol, Cortisone	<0.01%
17α-Oestradiol, 17β-Oestradiol, Prenenolone, Prenisone, Cholesterol	<0.0002%

alter internal sodium level can interfere with the accuracy in measurement of cation transport.

In addition to the steroids that might cross-react with the anti-digoxin antibody, digoxigenin bis- and mono-digitosixide and digoxigenin itself all react with the antibodies, whereas dihydrodigoxigenin and dihydrodigoxin metabolites in which the C22 is reduced show little cross reactivity [8].

2.5 Digoxin-like immunoreactivity in clinical settings

Plasma from normal subjects who are not on digoxin therapy has been shown to modestly inhibit ATPase activity [3]. In the mid sixties, when digoxin levels were measured by the inhibition of ^{86}rubidium uptake by human erythrocytes, false positive results were found in patients who had not taken digitalis compounds or other drugs known to interfere in the assay [5]. With some of the commercial immunoassay kits, false positive digoxin levels have been reported in renal failure patients who are not taking digoxin [15]. Similar false positive observations have also been reported in patients with liver impairment [16], in pregnancy [17], in hypertension [18], in amniotic fluid [7] and in cord blood [17]. Digoxin like

immunoreactivity observed in these studies can be due to either a specific compound or non-specific interference in the assay.

2.6 Presence of digoxin-like immunoreactive substance (DLIS)

The evidence for the presence of endogenous DLIS is supported by the studies on a circulating natriuretic factor involved in salt and fluid volume regulation [19]. This factor, termed third factor or natriuretic factor, has been extensively studied over the past two decades. Digoxin like immunoreactivity by RIA method was shown to be increased in a digoxin free animal when the extracellular fluid volume was expanded by saline infusion [20]. The factor has been partially purified by HPLC and it shows parallel antibody displacement responses in the RIA when compared with digoxin. The supposed natriuretic hormone has been isolated from plasma [21], placenta [22] and brain [23]. It cross reacts with digoxin antibody, inhibits [Na^+,K^+]-ATPase, and also causes natriuresis and diuresis [24].

2.7 Implications of digoxin-like immunoreactivity

The above observatic have r sed two

important issues related to the presence of digoxin-like immunoreactivity in human beings. One is the detrimental impact on the accuracy of digoxin measurement and the other is its patho-physiological implications.

2.7.1 Interference with digoxin measurement

Digoxin is routinely measured in serum or plasma by immunoassays. The therapeutic range for patients on digoxin treatment is 1.0-2.6 nmol/l. This narrow therapeutic range places rigorous demands on the accuracy and reproducibility of digoxin assays. Presence of DLIS in patients with renal failure, liver impairment, pregnancy and newborn infants makes the interpretation of plasma level difficult.

2.7.2 Patho-physiological implications

If the DLIS is a specific substance, it may have potential patho-physiological importance in renal failure, hepatic failure, newborn infants and pregnancy. The putative action of DLIS is to inhibit sodium transport by decreasing [Na^+,K^+]-ATPase activity and cause natriuresis. Patients with essential hypertension, renal failure, newborn infants and pregnant women are volume expanded. Therefore, the increase in DLIS in these patients may be linked to

volume expansion. It may also be involved in other salt and volume derangements.

It was reported that the concentration of DLIS in plasma more than doubled in response to both an intravenous salt load and a high-salt diet in patients with mild hypertension [25]. Correlation between the concentration of [Na^+,K^+] ATPase inhibitor in plasma and the mean atrial blood pressure of the normotensive and hypertensive individuals has also been demonstrated [26]. The physiological relevance of DLIS and its detection in other clinical conditions is the subject of research in the study of hypertension, salt and fluid regulation.

It has been suggested that DLIS is synthesized by the foetal-placental unit. This view is supported by i) high maternal serum concentration of DLIS in pregnancy, ii) production of DLIS decreases after birth as the foetal-adrenal glands start to involute [27], and iii) the identification of DLIS in human placental homogenates. However, there is a diverse range of substances yet to be identified as a modulator of the physiological reponses in hypertension of fluid homeostasis.

3. OBJECTIVE OF PROJECT

The cross reactivity of steroids and digoxin metabolites with the anti-digoxin antibody is well known and is usually stated for most commercial antisera. There are several explanations for these cross-reactions, but the two pertinent reasons are : firstly, the falsely high level of digoxin measurement is due to non-specific interference. Secondly, the falsely high level of digoxin measurement is due to the structurally related steroids or DLIS which react directly with the specific anti-digoxin antibodies.

Some compounds eg. lipids, fatty acids and proteins do not cross-react but nevertheless affect the radioimmunoassay (RIA) results by sequestering labelled digoxin to yield false-positive results. Organic solvents, pH, ions and proteins affect antibody binding in many radioimmunoassays. To distinguish between the specific and non-specific interferences in an antigen-antibody system is difficult. But usually, though not universally true, a parallel response with calculated degree of displacement can be used as a marker for the contribution of a specific interference.

While the conjugate is prepared by the

linkage through the carbohydrate moiety of digoxin to the bovine serum albumin, antibodies raised against this digoxin conjugate are directed mostly against the steroid moiety. As a result, cross reaction of the digoxin metabolites and many related steroids with the anti-digoxin antibody may be expected.

Although an endogenous DLIS has yet to be isolated and characterized, some steroid hormones or their intermediates have been proposed as likely candidates. The interactions of some common steroid hormones and intermediates which are raised in foetal cord blood, with the rabbit anti-digoxin antibodies are studied in an attempt to demonstrate if they could account for the falsely high level of digoxin-like immunoreactivity in the cord blood. The objectives of this project are :-

1. To measure the digoxin immunoreactivity of cord blood samples.

2. To study heat stability of DLIS as one of the physical properties of DLIS.

3. To study the concentration of steroids in cord blood and to see if this correlates with DLIS.

4. To examine the cross reactivity of steroids in digoxin radioimmunoassay.

5. To study the [Na^+,K^+]-ATPase inhibitory activity of cord blood extracts.

6. To study the inhibition of [Na^+,K^+]-ATPase by steroids.

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Digoxin RIA kits

Digoxin RIA kits were obtained from New England Nuclear (NEN), North Billerica, MA, USA

4.1.2 Steroid RIA kits

RIA coat-a-count kits for cortisol, oestradiol, progesterone, DHEAS, androstenedione, 17-OHP, total and free oestriol were obtained from Diagnostic Products Corporation (DPC), Los Angeles, USA

4.1.3 Chemicals

Cortisol, oestradiol, progesterone, DHEAS, androstenedione, 17-OHP, ouabain, adenosine 5'-triphosphate (sodium salt), dog kidney ATPase, phosphoenolpyruvate pyruvate kinase/lactate dehydrogenase, NADH, tris(hydroxymethyl)methylamine were obtained from Sigma Chemical Co., St. Louise, USA. Activated charcoal, sodium chloride, magnesium sulphate, ethylenediaminotetra-acetic acid disodium salt (EDTA), 1,2-di(2-aminoethoxy)ethane-NNN'N"-tetra-acetic acid (EGTA), N-tris(hydroxymethly)methyl-2-amino-ethanesulphonic acid (TES) and methanol were

obtained from British Drug House (BDH), Dorset, England. Sep-pak C_{18} cartridges were obtained from Waters Associates, Milford, Massachusetts, USA. Tri-level ligand control sera were obtained from Ciba-Corning Diagnostic Corp., USA.

4.1.4 Collection of blood samples

10-15 ml of cord blood was drawn from the umbilical cords of 29 neonates delivered vaginally. Blood was taken from the placental end of the cord after delivery. All were full term deliveries and none of the women were on digoxin therapy. The blood sample was allowed to clot by standing at room temperature for 2 hours. The blood was centrifuged at 1,800g for 5 minutes in a Beckman TJ-6 centrifuge and the serum was dispensed into 1 ml aliquots and stored at -30° C until assay.

Clotted blood was obtained from the Red Cross Blood Transfusion Centre and the sera pooled, irrespective of sex and age. They were used for the preparation of steroid free serum.

4.2 Methods

4.2.1 Digoxin assay

The [^{125}I] tracer is carried by histamine which is coupled to the terminal sugar of the tridigitoxose moiety. This tracer is claimed to exhibit high immunoreactivity and stability. The digoxin antibody was raised in rabbit and was pre-reacted with an antiserum to rabbit gamma globulin in phosphate buffer.

The recommended procedure was followed except the volume of the standards, samples, control serum, tracer and antiserum complex were reduced to 2/5 for sake of economy. This did not affect the accuracy and precision of the assay (Ho CS, personal communication). All standards and samples were assayed in duplicate and the mean values were taken. Duplicates with variation greater than 10% were repeated.

4.2.2 Radioimmunoassays of steroids

RIA for cortisol, oestradiol, progesterone, DHEAS, androstenedione, total and free oestriol and 17-OHP were performed essentially according to the

manufacturer's recommended procedure.

Briefly in these assays, the immobilized antibody reacted with [^{125}I]-labelled tracer and unlabelled analyte for a period of time. At the end of the incubation period, the supernatant containing unreacted analyte was aspirated and the amount of label bound to the antibody was counted in a gamma counter. All standards, controls and cord blood samples were assayed in duplicate.

As the concentrations of steroids in cord blood were expected to be high, cord blood sera were diluted with steroid free serum for the following assays :

Assays	Dilution
oestradiol	1:10
progesterone	1:50
DHEAS	1:3
total oestriol	1:10
free oestriol	1:10
17-OHP	1:10

In the androstenedione assay serum samples were extracted with diethyl ether prior to assay. The extract was redissolved in buffer (zero calibrator) after evaporating the ether in a vacuum oven.

Both free and conjugated oestriol were

measured in the determination of total oestriol. This was done by simultaneous incubation of tracer-enzyme solution which was freshly prepared by mixing the tracer solution with enzyme solution containing glucuronidase and sulphatase prior to assay.

4.2.3 [Na^+, K^+]-ATPase Inhibition Assay

ATPase activity was assessed by an enzyme coupled reaction using pyruvate kinase (PK) and lactate dehydrogenase (LD).

$$ATP \xrightarrow{ATPase} ADP + Pi$$

$$ADP + Phosphoenolpyruvate \xrightarrow{PK} Pyruvate + ATP$$

$$Pyruvate + NADH \xrightarrow{LD} Lactate + NAD$$

The ADP generated by ATPase reaction was used for the formation of pyruvate in the presence of phosphoenolpyruvate and pyruvate kinase. Lactate dehydrogenase then converts the pyruvate into lactate at the expense of NADH. The ATPase activity is therefore related to the decrease in NADH concentration ie. the decrease of absorbance at 340 nm.

4.2.3.1 Reagents for preincubation :-

1. Incubation buffer

100 mM NaCl, 4 mM $MgSO_4$ and 0.5 mM EDTA in 50 mM Tris buffer pH 7.4

2. Adenosine 5'-triphosphate solution

5 mM Adenosine 5'-triphosphate, sodium salt in the incubation buffer

3. [Na,K]-Adenosine 5'-triphosphatase solution

5 U of dog kidney [Na,K] ATPase was dissolved in 1 ml of 10 mM Tris buffer containing 0.5 mM EDTA. This stock solution was separated into 50 µl aliquots and kept at -70° C until use. 950 µl of incubation buffer was added to the 50 µl [Na,K] ATPase stock aliquot before use.

4. Calibration standards

5, 10, 20, 30, 50, 70 nM ouabain standards were prepared in incubation buffer.

4.2.3.2 Reagent for [Na,K]-ATPase activity assay :-

1. 128 mM TES-Tris buffer pH7.4

2. Na-K solution

107 mM KCl, 533 mM NaCl, 32 mM $MgSO_4$ and 27 mM EGTA in 128 mM TES-Tris buffer pH 7.4

3. Adenosine 5'-triphosphate (ATP) solution

96 mM Adenosine 5'-triphosphate (sodium salt) in 128 mM TES-Tris buffer pH 7.4

4. Phosphoenolpyruvate (PEP) solution

38 mM Phosphoenolpyruvate in 128 mM TES-Tris buffer pH 7.4

5. PK-LD solution

55 µl of pyruvate kinase/lactate dehydrogenase solution was diluted to 1 ml with 128 mM TES-Tris buffer pH 7.4

6. NADH solution

18 mM NADH in 128 mM TES-Tris buffer pH 7.4

7. Working solution

The following working solution was prepared fresh before assay :-

Na-K solution	4800 µl
ATP solution	800 µl
PEP solution	800 µl
NADH solution	800 µl
PK-LD solution	800 µl

4.2.3.3 Assay procedure

1. Sep-pak C_{18} cartridge was pre-wetted with 2 ml of methanol and then washed with 10 ml of distilled water.

2. 200 µl of cord serum sample was injected at one end of the cartridge and it was allowed to stand for 3 minutes.

3. 20 ml of distilled water in syringe was pumped through the cartridge to wash away the high polarity compounds.

4. Low polarity compounds were eluted with 2 ml of methanol and the eluate was collected in an Eppendorf tube. (Sep-pak C_{18} cartridge was then washed with 20 ml of distilled water for reuse. Each cartridge was used for the preparation of five cord serum samples.)

5. The eluates were evaporated to dryness under negative pressure at 40° C.

6. Once dry, the residue was resuspended in 200 µl of incubation buffer and vortex mixed.

7. 100 µl each of ouabain standards or serum sample extracts, 50 µl ATP solution and 50 µl of [Na,K] ATPase solution were added to an Eppendorf tube, vortex mixed and incubated at 37° C for 2 hours.

8. After incubation, the above solution was transferred to a Cobas-Bio centrifugal analyzer. The enzyme reaction was started by the addition of working solution mixture of reagent 7. The absorbance at 340 nm was followed kinetically.

9. The Cobas-Bio programme parameters :-

a.	units	U/L
b.	calculation factor	4013
c.	standard 1 conc	0
d.	standarg 2 conc	0
e.	standard 3 conc	0
f.	limit	0
g.	temperature (deg.C)	37.0
h.	type of analysis	2
i.	wavelength (nm)	340
j.	sample volume (µl)	40
k.	diluent volume (ul)	50
l.	reagent volume (µl)	50
m.	incubation time (sec)	0
n.	start reagent volume (µl)	0
o.	time of first reading (sec)	300.0
p.	number of readings	30

q. blanking mode 0
r. printout mode 1

10. The inhibition caused by the unknown was determined from the calibration curve and expressed as ouabain equivalent.

4.2.4 Preparation of steroid free serum

10 g of activated charcoal was added to 200 ml of serum, and the contents were stirred with a magnetic stirrer for 2 hours at 4°C. The serum was centrifuged at 4500 rpm (7,000g) in a Beckman J-6B centrifuge at 4°C for 15 minutes. The supernatant was removed and passed through 0.45 um Millipore filter. The concentration of digoxin and steroids of interest were measured in the pooled serum before and after the charcoal treatment.

4.2.5 Precision and detection limit

The detection limit of digoxin RIA was studied by analyzing ten samples of zero calibrator obtained from RIANEN (New England Nuclear, North Billerica, MA, USA). The value of mean+2SD was taken as the sensitivity of digoxin assay based on 95%

confidence limit.

Precision of the ATPase inhibition method was determined by eight analysis of a pooled cord blood serum samples. Cord blood sera were prepared by passing through the Sep-pak C_{18} cartridge. The methanol eluates were assayed for the ATPase inhibition. The value of eight separate runs of the pooled serum were used for the calculaton of inter-assay precision of the [Na,K] ATPase assay.

Inter-assay precisions of cortisol, oestradiol and progesterone radioimmunoassays were determined by multiple analysis of Ciba-Corning Tri-level Ligand Control sera A, B and C. Each control sample was analyzed in twelve separate assays for cortisol and estradiol, and eleven times for progesterone. Single level control serum was provided with the digoxin assay kit was assayed in nine separate runs. The coefficients of variation (CV) were calculated as below :

$$CV = \frac{\text{Standard deviation}}{\text{Mean}} \times 100\ \%$$

4.2.6 Effect of heating on digoxin-like immunoreactivity and steroid levels

Cord blood sera and charcoal treated pooled serum were diluted 1:3 with deionised water, and heated at 100°C for 5 minutes in a boiling water bath. Water was used to decrease the ionic strength and protein concentration of the serum samples to prevent protein coagulation upon heating [28]. Heated sera were allowed to cool and then assayed for digoxin, oestradiol and cortisol levels on the same day.

4.2.7 Cross reactivity of steroids in digoxin RIA

Since all steroids studied are naturally occurring intermediates in human serum, they may contribute to the digoxin-like immunoreactivities observed in newborns and pregnant women. The cross reactivity of cortisol, oestradiol, progesterone, 17-OHP, androstenedione, DHEAS and oestriol were examined.

In order to simulate the physiological state of the steroids measured and to preserve the matrix integrity for immunoassay, a stock solution of steroids dissolved in methanol were diluted serially in steroid

free serum. These were then assayed for DLIS using the digoxin RIANEN kit. The percentage binding of digoxin was plotted against the concentration of the competing steroid. The respective dose response curve is compared against the standard curve of digoxin.

The concentration of the steroid causing 50% displacement (D_{50}) of digoxin tracer was used to determine percentage cross reactivity. The cross reactivity of steroids was calculated by dividing the D_{50} (molar) value of digoxin by the D_{50} (molar) value of the respective steroid and was expressed as a percentage.

$$\text{Cross reactivity} = \frac{D_{50} \text{ of Digoxin (molar)}}{D_{50} \text{ of steroid (molar)}} \times 100\%$$

4.2.8 Digoxin immunoreactivity and ATPase inhibitory reactivity of simulated cord blood samples

Four serum samples were prepared by adding various concentrations of steroids to steroid free serum to mimic cord blood samples. Four typical cord blood samples that had a high DLIS level (sample C43), intermediate level (samples C31 and C48) and low level (sample C14) were selected. These four samples also had a higher concentration of progesterone. The

concentration of cortisol, oestradiol, DHEAS, progesterone, 17-OHP, androstenedione and free oestriol in these samples were obtained and stock solution of steroids were added to charcoal treated pooled serum to simulate the four cord blood samples and they were labelled S14, S31, S43 and S48.

Four additional samples were prepared based on the calculated mean concentrations of steroids in the 29 cord bloods analyzed. Sample A had the steroid concentrations at 2SD above the means. Sample C had the steroid concentrations similar to their mean concentrations in the cord blood. The steroid concentrations of samples B and D were half those of samples A and C respectively.

All the simulated cord blood samples were assayed for digoxin-like immunoreactivity before and after Sep-pak C_{18} chromatography. Their ATPase inhibitory activities were also studied.

4.2.9 Inhibition of $[Na^+,K^+]$-ATPase by steroids

The same series of steroids mentioned in section 4.2.2 were assayed for their ability to inhibit ATPase. Serial dilutions of the steroids were made in

50mM tris buffer pH 7.4. They were then assayed directly by incubation with dog kidney ATPase for 2 hours at 37° C. The residual ATPase activity was then assayed on the Cobas-Bio centrifugal analyzer by the coupled reaction mentioned before.

4.2.10 Procedure for calculation of unknown digoxin and steroid concentrations in RIA

The net counts for each set of standards and samples were obtained by subtracting from each the average background and non-specific binding counts. The counts were then averaged for each set of duplicate and they were expressed as a percentage of the average net count for the zero standard (where B=average net count of samples, B_0=average net count of zero standard).

$$\% B/B_0 = \frac{\text{average net count of standard or sample}}{\text{average net count of zero standard}} \times 100$$

Logit-log plot with $\%B/B_0$ against concentration for each standard was plotted. The concentration of samples and controls were determined by intrapolation from the standard curve.

4.2.11 Statistical analysis

The difference between digoxin-like immunoreactivity before and after heating test were determined by Student's t-test for paired results.

The relationship between digoxin-like immunoreactivity and the steroids was determined by linear regression analysis. The correlation coefficient measured the extent of association between the digoxin-like immunoreactivity and steroid concentration.

The multiple correlation between digoxin-like immunoreactivity and the combined effect of steroids were studied by multiple linear regression analysis.

The test of the significance of multiple correlation was studied by the analysis of variance methods using F distribution test.

Linear regression analysis and multiple linear regresssion analysis were done using computing software Abstat Release 3.01 (Anderson-Bell Co.,USA). 95% confidence limit was taken as the significance value.

5. RESULTS

5.1 Precision and detection limit of assays

Inter-assay precision of the radioimmunoassays represented as coefficient of variation (CV) are shown in Table 2. Highest CV was found in the assay of progesterone. The CV varied from 9.4% to 13.3%. Digoxin assay had a CV of 4.4% showing a good precision of the digoxin RIA.

The detection limit of the RIANEN digoxin assay was found to be 0.13 nmol/l.

5.2 DLIS in cord blood samples

29 cord blood samples were analyzed for the digoxin level using the digoxin radioimmunoassay. The mean level of DLIS was found to be 0.91 nmol/l (range 0.50-1.37 nmol/l) (Figure 2).

Table 2 Precision of radioimmunoassay for the digoxin and steroid assays

Analytes	Control	Mean	N	S.D.	CV
Cortisol	1	182 nmol/l	12	12.8	7.0%
	2	549 nmol/l	12	14.4	2.6%
	3	1316 nmol/l	12	46.6	3.5%
Oestradiol	1	3.13 nmol/l	12	0.20	6.4%
	2	20.72 nmol/l	12	2.32	11.2%
	3	66.28 nmol/l	12	5.62	8.5%
Progesterone	1	0.41 nmol/l	11	0.55	13.3%
	2	1.88 nmol/l	11	0.21	11.2%
	3	3.63 nmol/l	11	0.34	9.4%
17α-hydroxy-progesterone *	1	1.6 nmol/l	20	0.079	4.8%
	2	3.3 nmol/l	20	0.15	4.5%
	3	15.1 nmol/l	20	0.82	5.4%
	4	24.5 nmnol/l	20	1.39	5.7%
Androstenedione *	1	0.49 nmol/l	25	0.042	8.6%
	2	0.735 nmol/l	25	0.063	8.6%
	3	1.295 nmol/l	25	0.119	9.2%
Free Oestriol *	1	15.2 nmol/l	98	1.53	10.0%
	2	43.0 nmol/l	98	2.74	6.4%
	3	96.4 nmol/l	98	5.31	5.5%
Total Oestriol *	1	93.6 nmol/l	20	7.6	8.2%
	2	308.6 nmol/l	20	17.3	5.6%
	3	613.7 nmol/l	20	36.1	5.9%
Digoxin	1	3.8 nmol/l	9	0.17	4.4%

* The inter-assay CV are quoted from DPC Coat-a-count kits

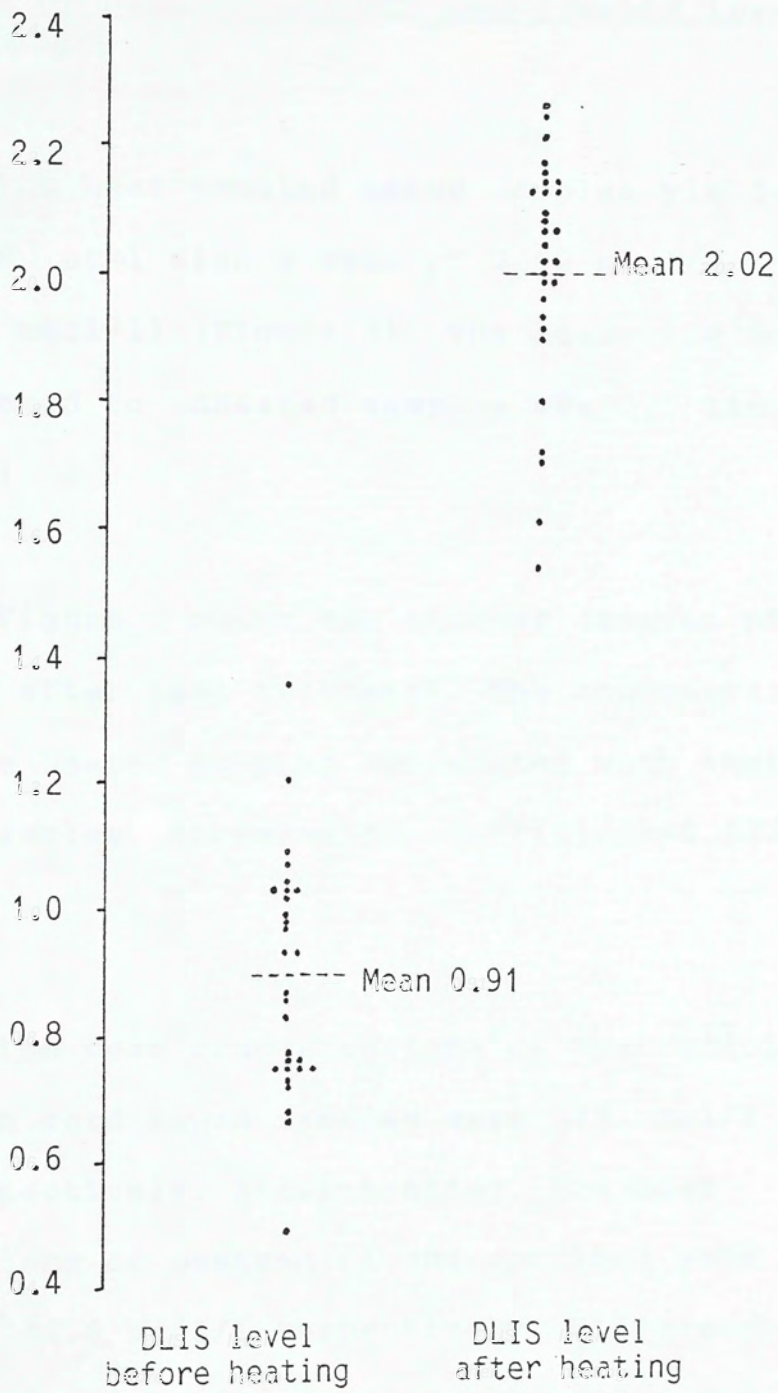


Figure 2. DLIS level in cord blood before and after heating

5.3 Effect of heating on DLIS and steroid levels in cord blood

The heat treated serum samples yielded even higher DLIS level with a mean of 2.02 nmol/l (range 1.567-2.28 nmol/l) (Figure 2). The mean rise in DLIS level compared to unheated samples was 2.3 times (range 1.6 to 3.1).

Figure 3 shows the scatter diagram of DLIS before and after heat treatment. The concentration of DLIS in the heated samples correlated with that of the unheated samples (correlation coefficient=0.537, $p<0.01$).

The mean concentrations of oestradiol and cortisol in cord blood samples were 275 nmol/l and 49.6 nmol/l respectively. After heating, the mean concentrations of oestradiol and cortisol were 243 nmol/l and 44.8 nmol/l respectively. Difference between the unheated and heated samples were not significant. Figure 4 shows the scatter diagram for oestradiol and cortisol before and after heat treatment.

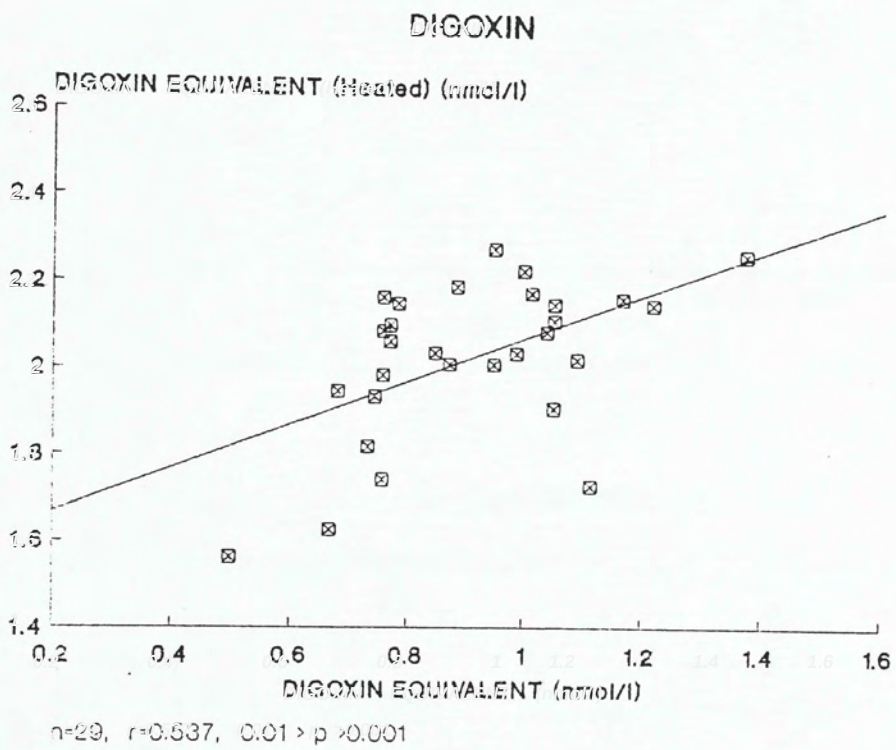


Figure 3. Correlation between DLIS in cord blood before and after heating

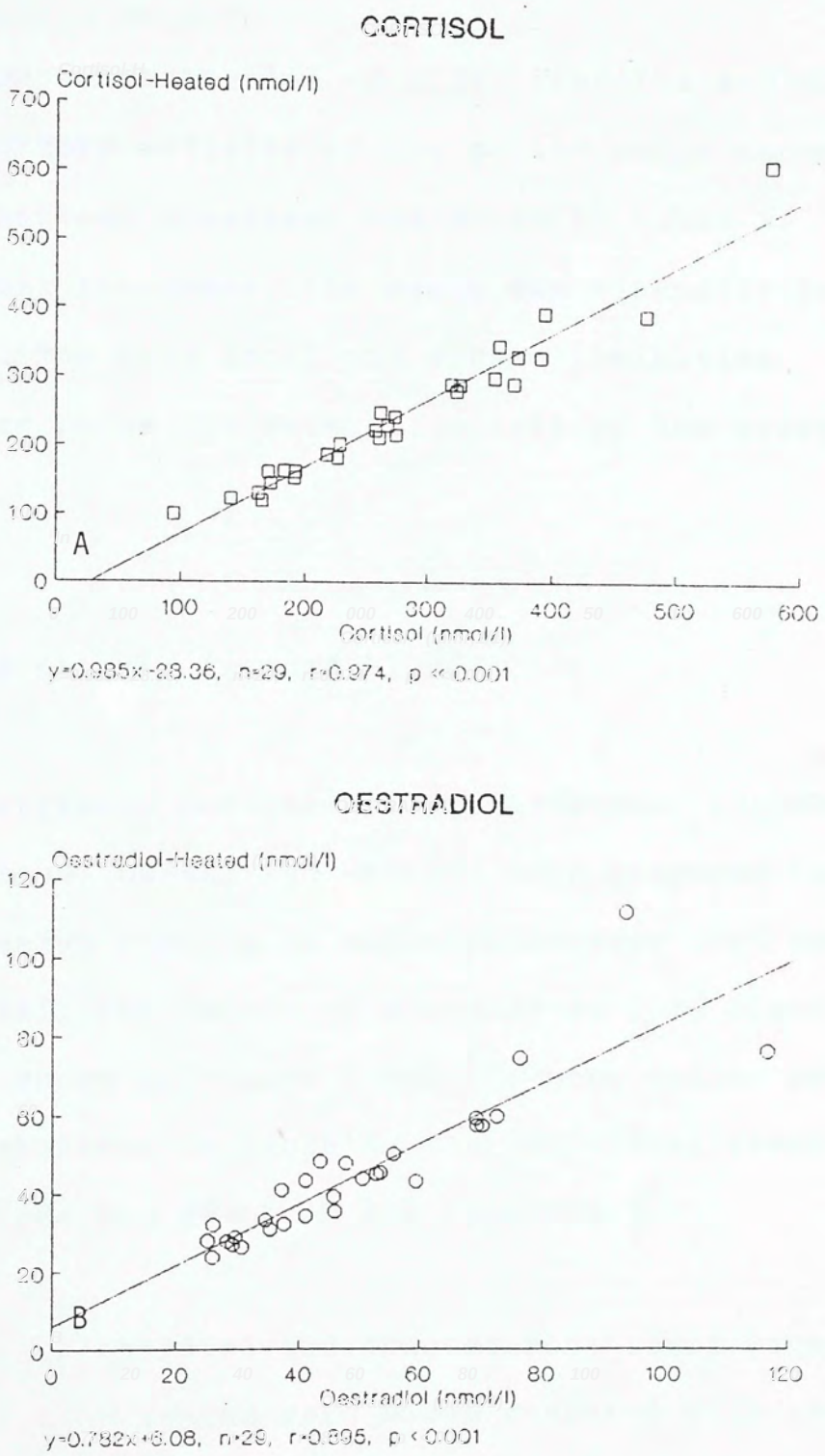


Figure 4. Correlation between cortisol (panel A) and oestradiol (panel B) in cord blood before and after heating

5.4 Effect of charcoal treatment on serum concentrations of steroids and DLIS

The concentration of DLIS, steroids and the ATPase inhibitory activity of the pooled serum before and after charcoal treatment are shown in table 3. After charcoal treatment, the serum was virtually free of steroids. The DLIS level and ATPase inhibition activity were below the detection limit of the assay.

5.5 Steroid pattern in cord blood

Cortisol, oestradiol, progesterone, 17-OHP, androstenedione, DHEAS, and estriol were measured in 29 cord blood serum samples by radioimmunoassay (DPC coat-a-count tubes). The levels of steroids in cord blood samples are shown in Figure 5 and the mean values and range are tabulated in Table 4. The individual results of the steroids are given in the appendix I.

The oestradiol and progesterone level were particularly high in the cord blood compared with the adults. Progesterone and total oestriol in cord blood were found to be 1000-2000 times higher than male adult level. Progesterone was about 20 times higher than the luteal phase in the adult female. Oestradiol levels

Table 3 Concentration of DLIS, steroids and ATPase inhibitory activity in pooled serum before and after charcoal treatment

Analytes	Pooled Sera	Steroid free serum
Digoxin	<0.13 nmol/ml	<0.13 nmol/ml
Cortisol	341 nmol/l	<27.6 nmol/l
Progesterone	1.66 nmol/l	<0.3 nmol/l
Oestradiol	117 pmol/l	<70 pmol/l
Androstenedione	6.30 nmol/l	<0.35 nmol/l
17-OHP	4.39 nmol/l	<0.3 nmol/l
Total Oestriol	<35 nmol/l	<35 nmol/l
Free Oestriol	<3.5 nmol/l	<3.5 nmol/l
ATPase Inhibition (Ouabain Equivalent)	32 nmol/l	<5 nmol/l

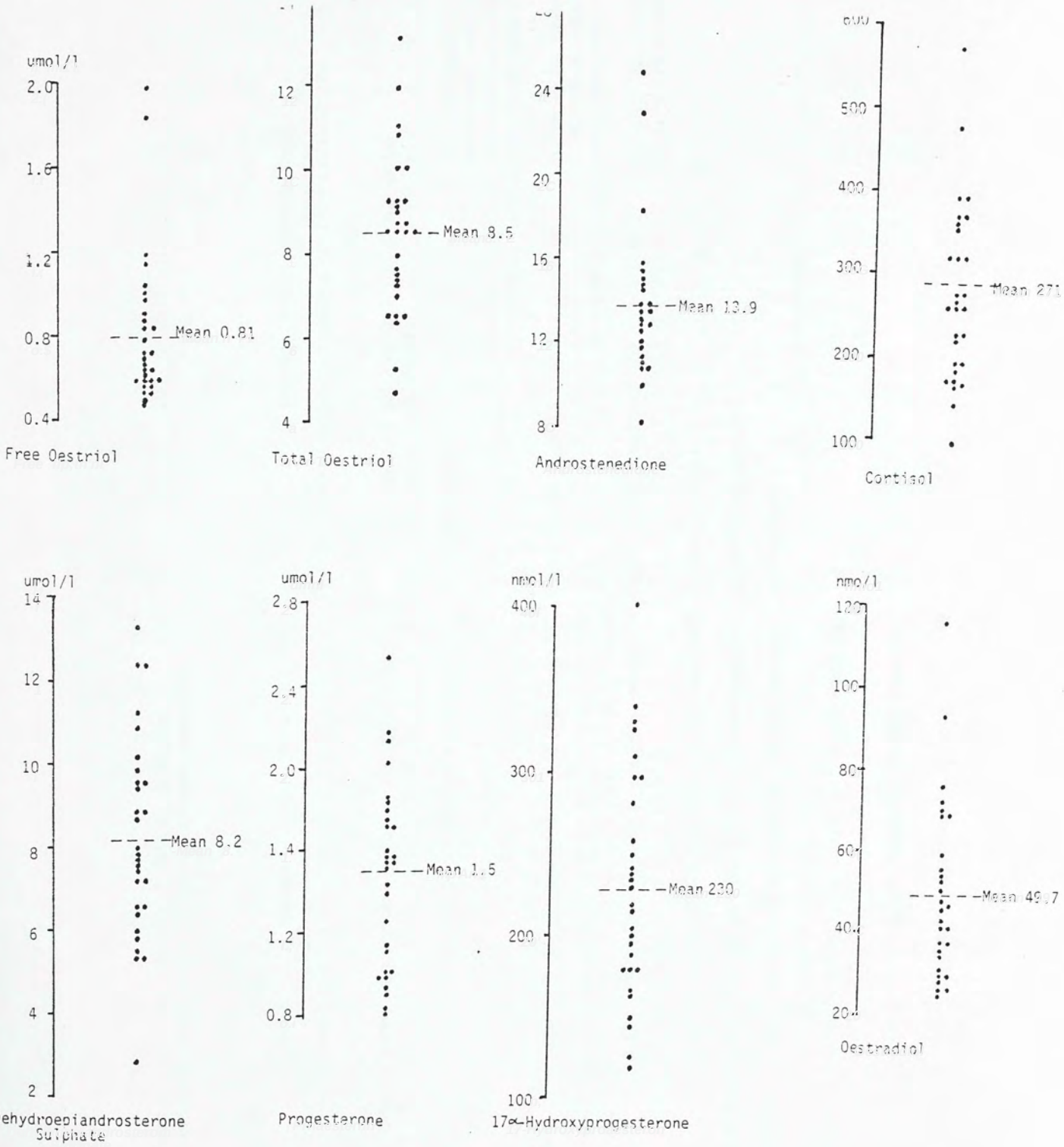


Figure 5. Concentration of steroids in cord blood

Table 4 Concentration of steroids and digoxin-like immunoreactive substance in cord blood samples

Analytes	N	Range (mean ± 2 SD)	Mean	Normal Adult Range [29]	
				Male	Female
Cortisol	29	64 - 486 nmol/l	275 nmol/l	138-635 nmol/l at 0800h <0.5 of 0800h at 2000h	as male
Oestradiol	29	7.00-92.25 nmol/l	49.7 nmol/l	29-132 pmol/l	Follicular: 37-330 pmol/l Mid-cycle: 367-1835 pmol/l Luteal: 184-881 pmol/l Pregnant(term):446-1031 pmol/l
Progesterone	29	0.60-2.39 µmol/l	1.49 µmol/l	0.38-0.95 nmol/l	Follicular: 0.06-2.86 nmol/l Luteal: 1.91-95.4 nmol/l Pregnant(3rd trim): 254-636 nmol/l
17-OHP	29	90-370 nmol/l	230 nmol/l	0.61-5.45 nmol/l	Follicular: 0.61-2.42 nmol/l Luteal: 2.42-9.10 nmol/l
DHEAS	29	3.44-13.00 µmol/l	8.22 µmol/l	5.2-8.7 µmol/l	2.1-8.8 µmol/l
Androstenedione	24	6.60-21.25 nmol/l	13.93 nmol/l	2.86-4.60 nmol/l	3.94-6.60 nmol/l
Total Oestriol	29	4.77-12.27 µmol/l	8.52 µmol/l	<7 nmol/l	<7 nmol/l Pregnant(40wks): 278-1215 nmol/l
Free Oestriol	29	0.11-1.51 µmol/l	0.81 µmol/l		
DLIS	29	0.53-1.29 nmol/l	0.91 nmol/l		

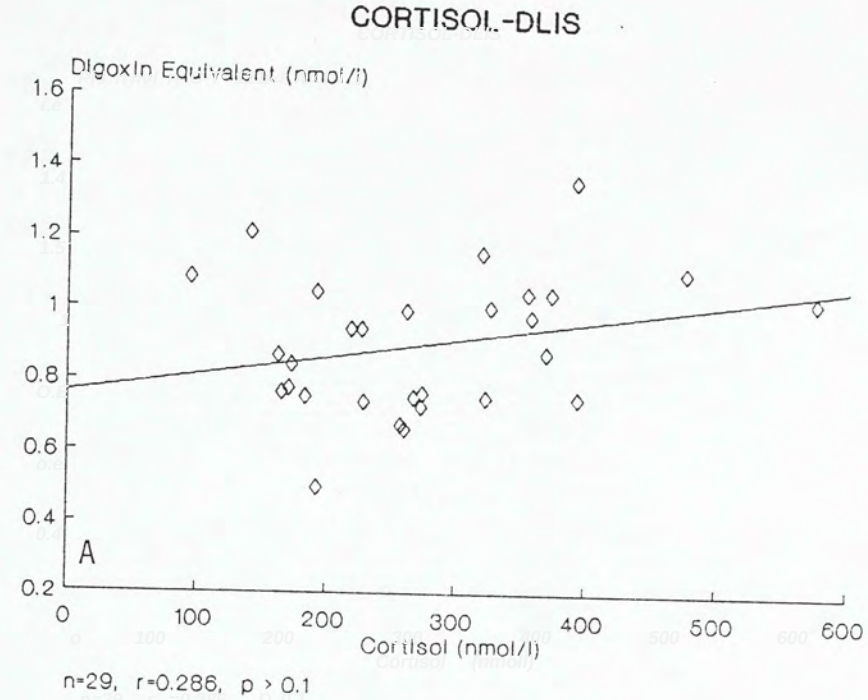
were about 200 to 1000 times higher than the adult male and about 5 times higher than that in luteal phase in female. The 17-OHP was about 10 times higher than that in the adult male, while the cortisol and androstenedione levels were comparable to the adult level.

5.6 Correlation between DLIS and concentration of steroids in cord blood

The scatter diagrams of digoxin-like immunoreactivity (before and after heat treatment) against the appropriate steroids are shown in Figures 6a-6h.

Statistical analysis (Tables 5a and 5b) showed significant correlations of DLIS with progesterone, 17-OHP and oestradiol in the unheated samples. Heat treatment of cord serum samples yielded significant correlation between DLIS and progesterone, androstenedine, 17-OHP and free oestriol.

Although oestradiol correlated with DLIS in the unheated cord blood, no correlation was found in the heated sample. On the other hand, androstenedione showed a significant correlation with the heated



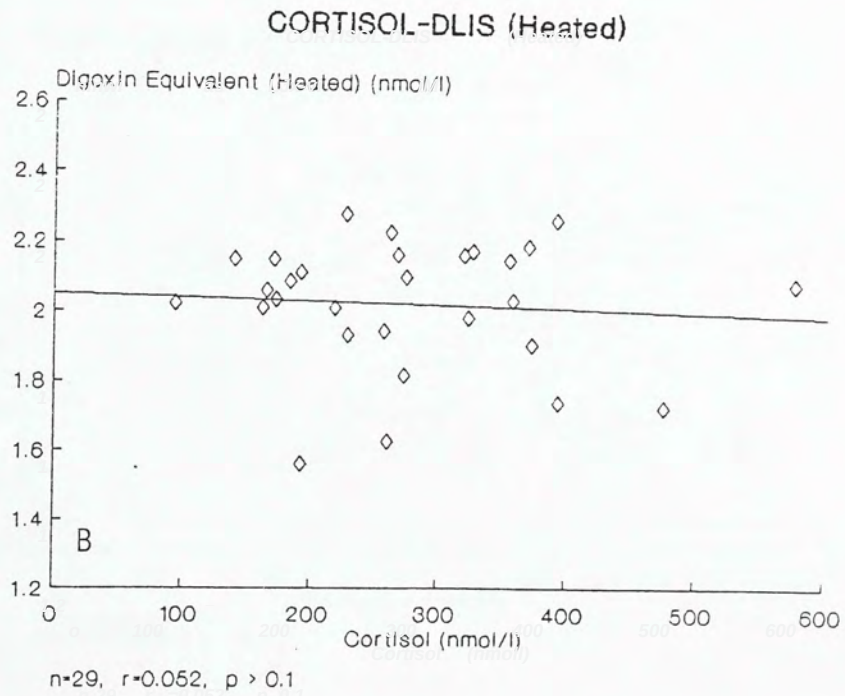


Figure 6a. Correlation between cortisol and DLIS in cord blood before heating (panel A) and after heating (panel B)

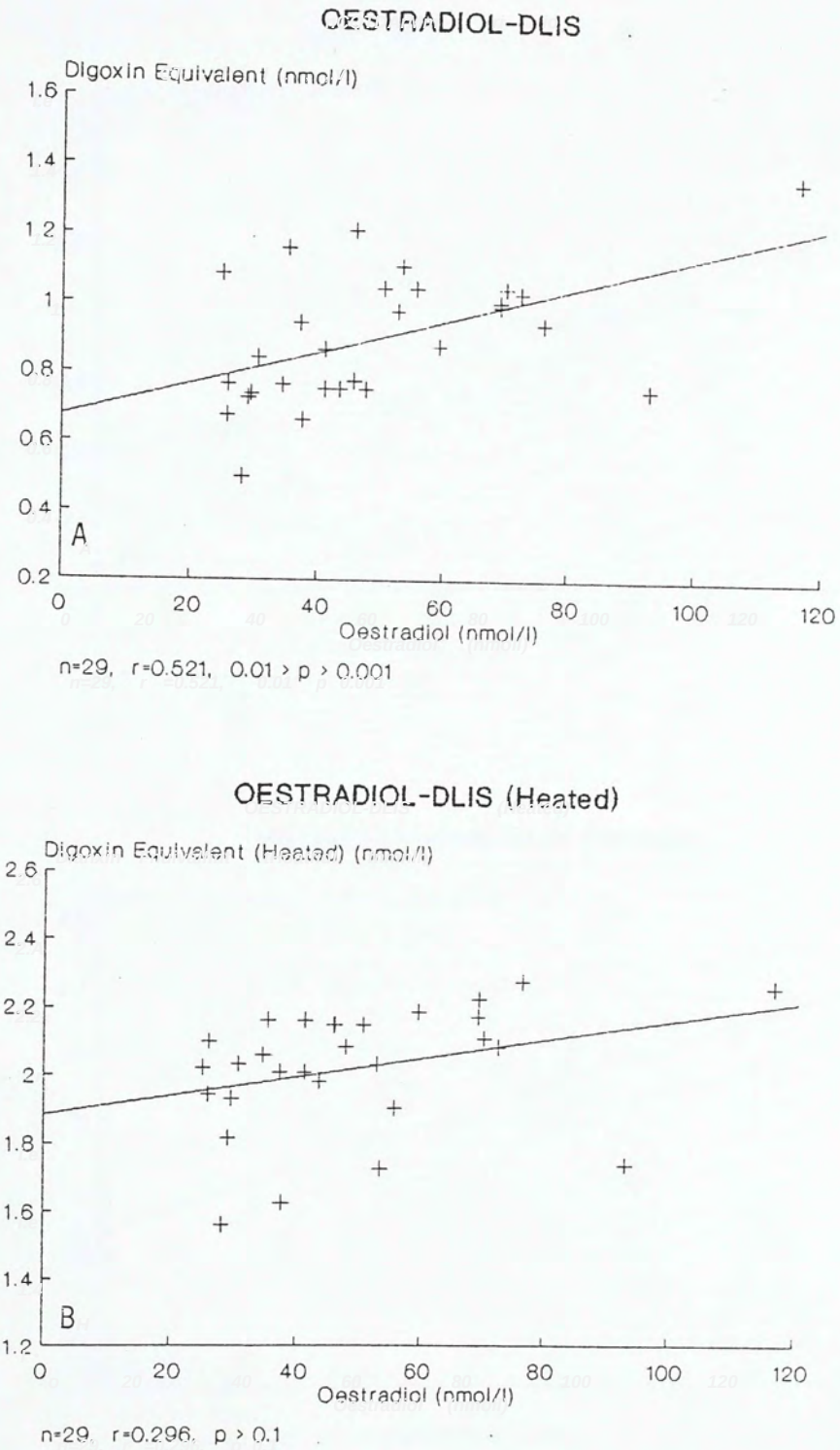


Figure 6b. Correlation between oestradiol and DLIS in cord blood before heating (panel A) and after heating (panel B)

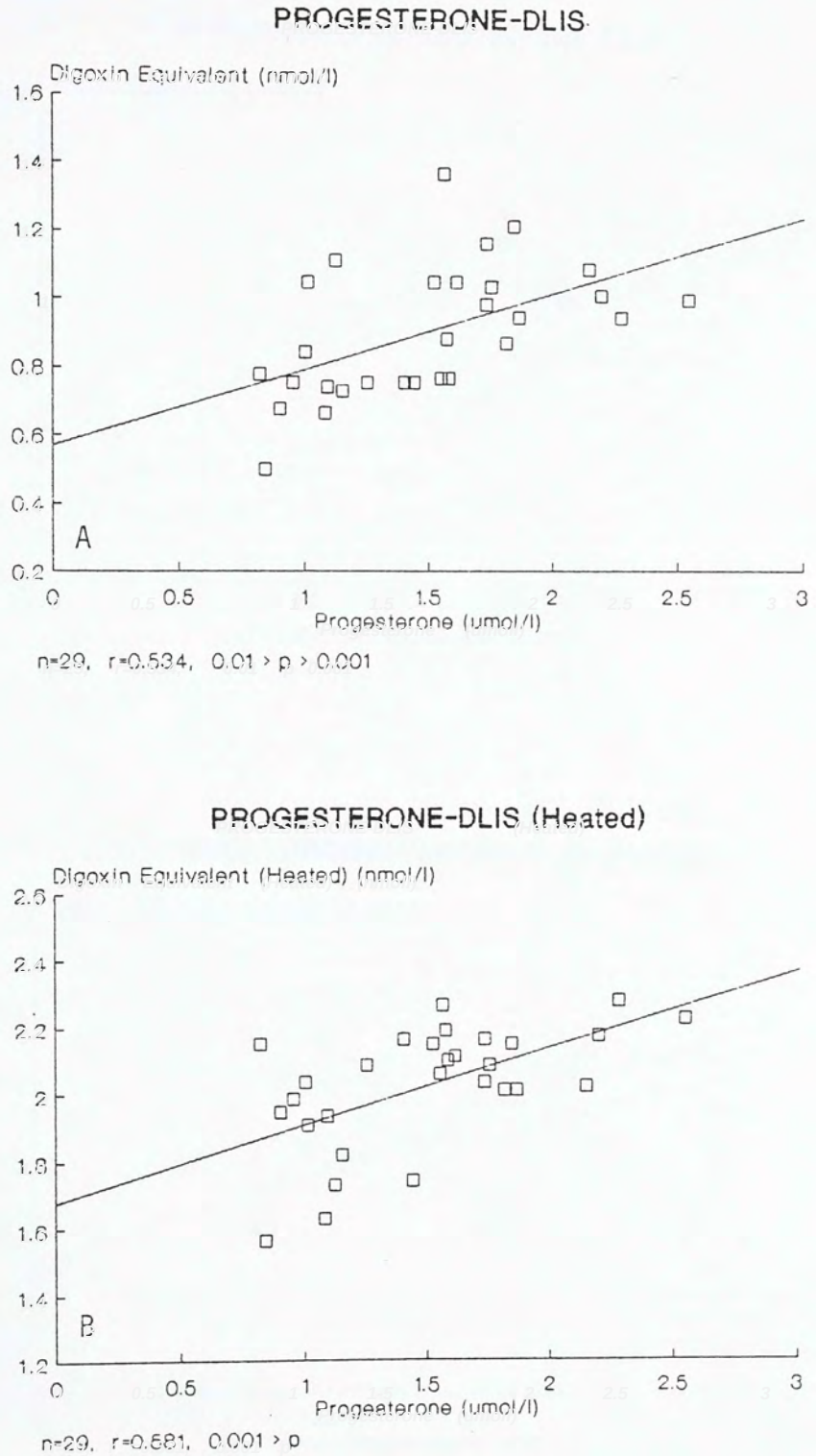
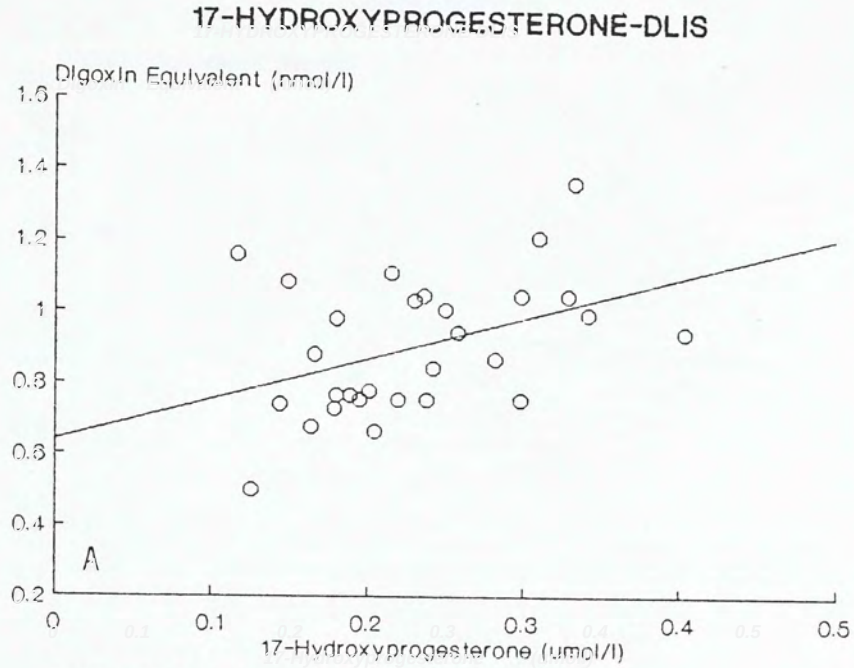


Figure 6c. Correlation between progesterone and DLIS in cord blood before heating (panel A) and after heating (panel B)



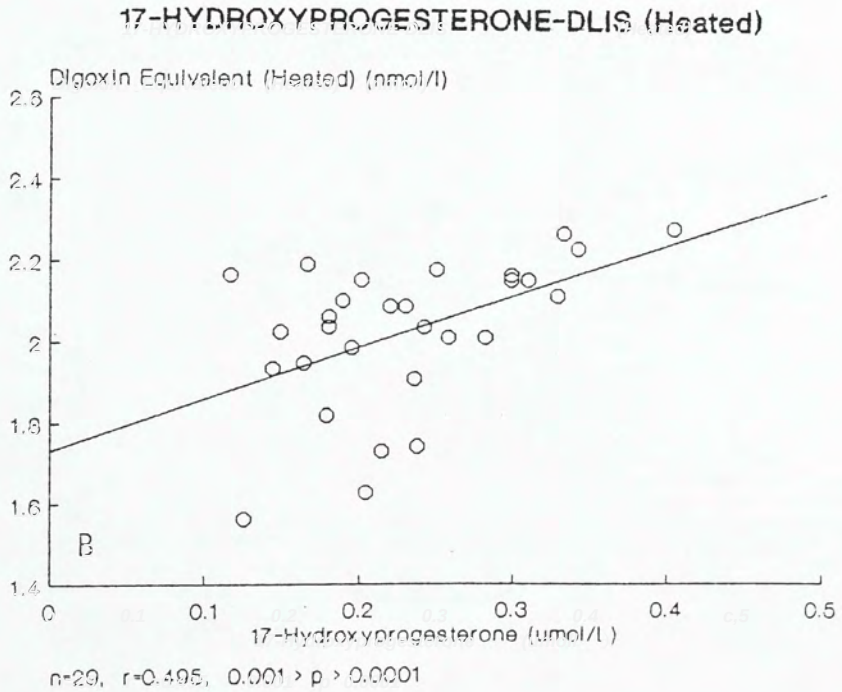
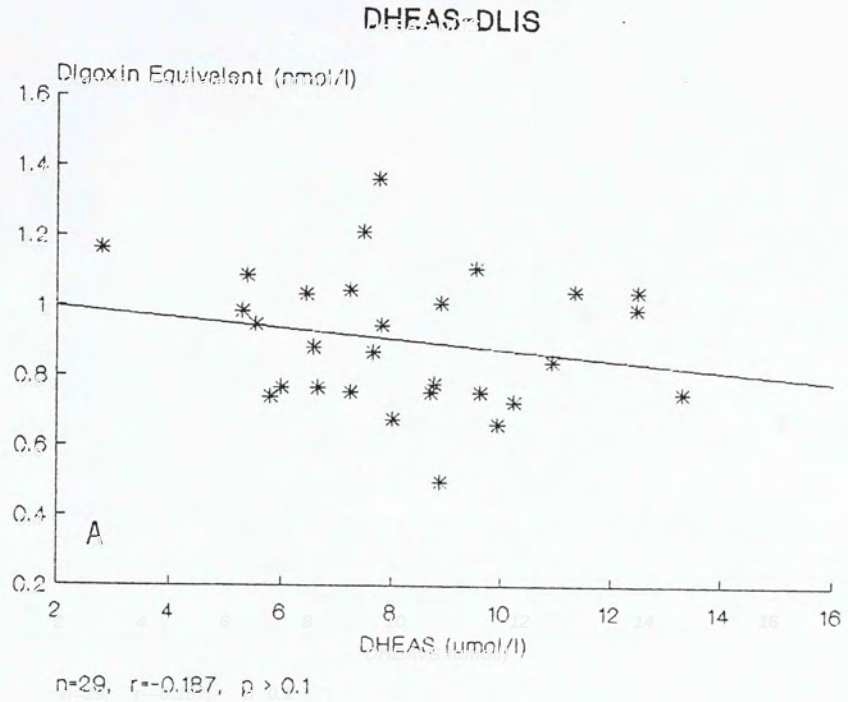


Figure 6d. Correlation between 17∝-hydroxyprogesterone and DLIS in cord blood before heating (panel A) and after heating (panel B)



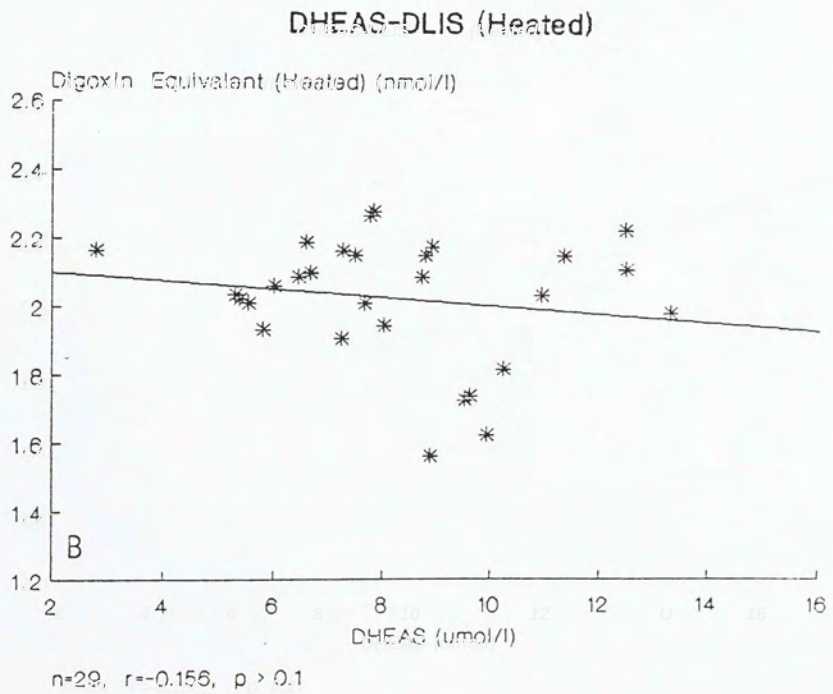
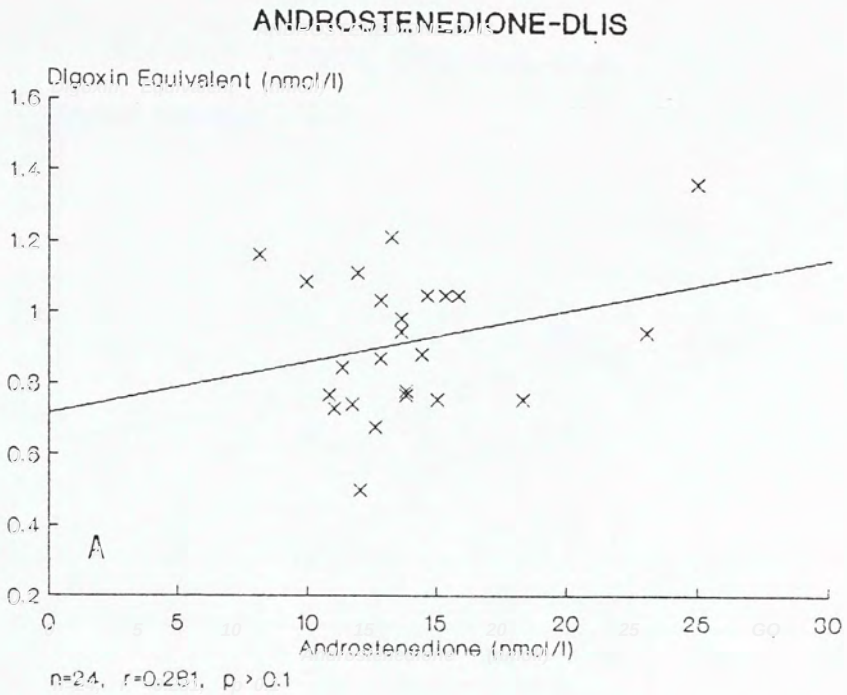


Figure 6e. Correlation between DHEAS and DLIS in cord blood before heating (panel A) and after heating (panel B)



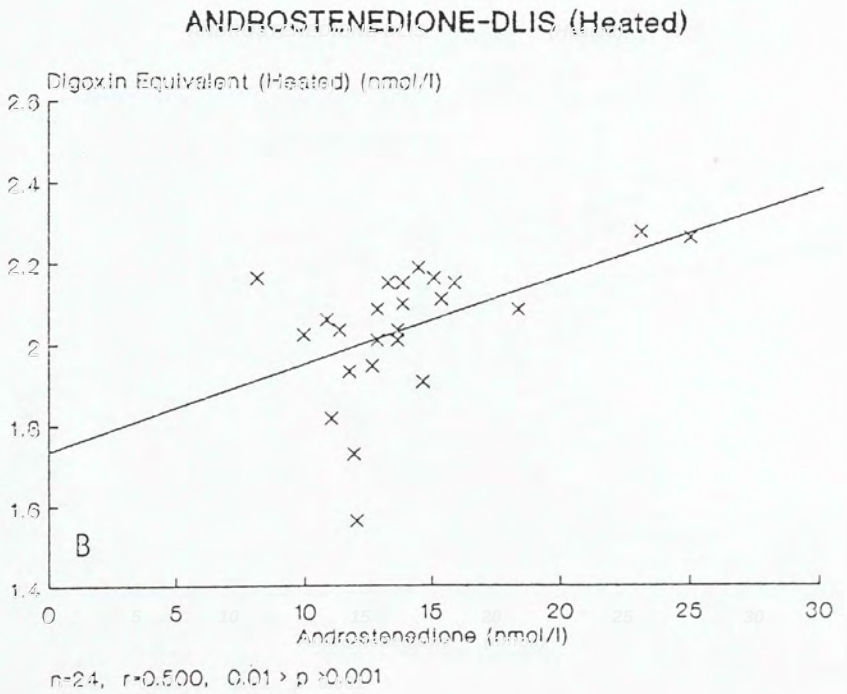
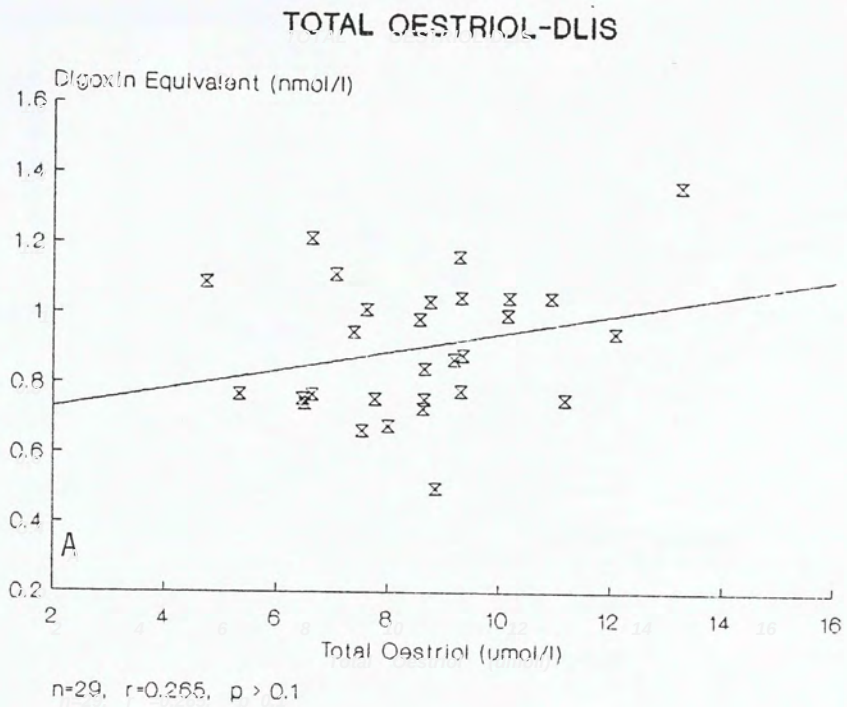


Figure 6f. Correlation between androstenedione and DLIS in cord blood before heating (panel A) and after heating (panel B)



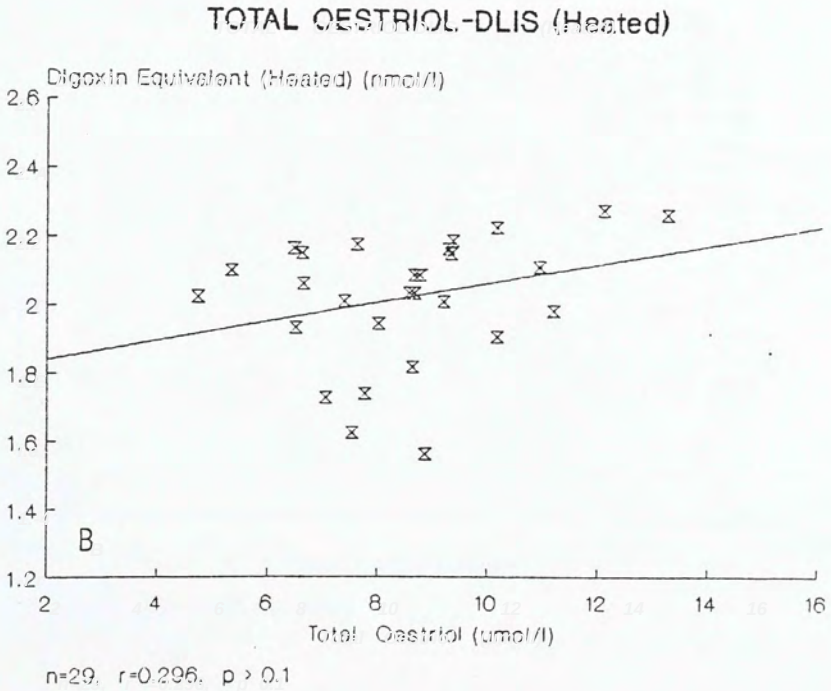
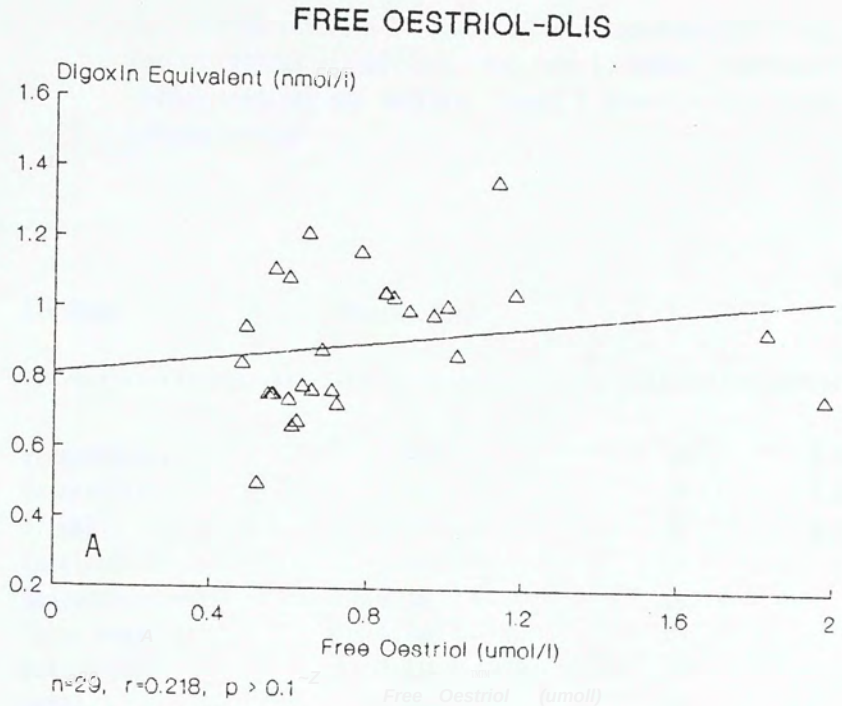


Figure 6g. Correlation between total oestriol and DLIS in cord blood before heating (panel A) and after heating (panel B)



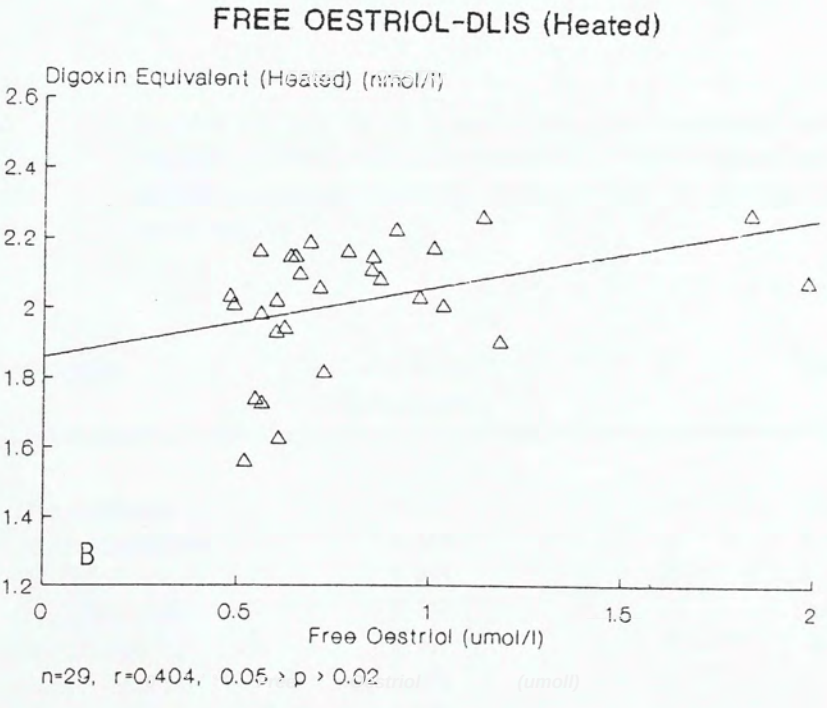


Figure 6h. Correlation between free oestriol and DLIS in cord blood before heating (panel A) and after heating (panel B)

Table 5a Pairwise correlation of digoxin-like immunoreactivity against the concentrations of cortisol, oestradiol, DHEAS, progesterone, 17-OHP, androstenedione and oestriol (total & free) in cord blood serum before heating

Steroids	Correlation	N	Significance Coefficients Level
Progesterone	0.534	29	0.01>p>0.001
Oestradiol	0.521	29	0.01>p>0.001
17-OHP	0.430	29	0.02>p>0.01
Cortisol	0.386	29	p>0.1
Androstenedione	0.381	24	p>0.1
Total Oestriol	0.365	29	p>0.1
Free Oestriol	0.218	29	p>0.1
DHEAS	-0.187	29	p>0.1

Table 5b Pairwise correlation of digoxin-like immunoreactivity against the concentrations of cortisol, oestradiol, DHEAS, progesterone, 17-OHP, androstenedione and oestriol (total & free) in the heat treated cord serum samples

Steroids	Correlation Coefficients	N	Significance Level
Progesterone	0.581	29	0.001>p
Androstenedione	0.500	24	0.01>p>0.001
17-OHP	0.495	29	0.01>p>0.001
Free Oestriol	0.404	29	0.05>p>0.02
Oestradiol	0.337	29	0.1>p>0.05
Total Oestriol	0.286	29	p>0.1
DHEAS	-0.156	29	p>0.1
Cortisol	0.052	29	p>0.1

digoxin-like immunoreactivity but not with the unheated one.

Correlation between DLIS and cortisol, DHEAS or total oestriol in cord blood before and after heat treatment were not significant.

There was a negative correlation between DHEAS and DLIS but this was not statistically significant.

Multiple regression analysis of the digoxin-like immunoreactivity with the steroids determined yielded a multiple correlation coefficient of 0.824. By the analysis of variance, the combined effect of the steroids included had a significant correlation with the measured digoxin level in cord blood. 68% of the observed variation in DLIS in cord blood could be explained by the combined effect of the steroids (R^2=0.679). As shown in Table 6a, oestradiol, progesterone and 17-OHP are relatively important in explaining the level of digoxin-like immunoreactivity with their partial correlation coefficient of 0.628, 0.523 and 0.422 respectively. The highest standardized partial regression coefficient of 1.147 was obtained between oestradiol and digoxin-like immunoreactivity.

Table 6a Partial multiple linear regression coefficients for digoxin-like immunoreactivity in respect of cortisol, oestradiol, progesterone, 17-OHP, DHEAS, androstenedione and oestriol (total & free) levels (N=24) before heat treatment

Steroids	Correlation with DLIS	Standardized Regression Coefficient
Cortisol	0.352	-0.055
Oestradiol	0.628	1.147
Progesterone	0.533	0.196
17-OHP	0.422	0.272
DHEAS	-0.137	-0.115
Androstenedione	0.381	-0.710
Total Oestriol	0.301	-0.190
Free Oestriol	0.148	0.011

Multiple correlation coefficient = 0.824

F(8,15) = 3.96 $0.01 < p < 0.05$

Intercept = 0.894

Table 6b Partial multiple linear regression coefficient for digoxin-like immunoreactivity after heating in respect of cortisol, oestradiol, progesterone, 17-OHP, DHEAS, androstenedione and oestriol (total & free) levels (N=24)

Steroids	Correlation with Heated DLIS	Standardized Regression Coefficient
Cortisol	0.002	-0.158
Oestradiol	0.484	0.289
Progesterone	0.551	0.218
17-OHP	0.520	0.300
DHEAS	-0.169	-0.300
Androstenedione	0.500	0.162
Total Oestriol	0.297	0.025
Free Oestriol	0.356	-0.042

Multiple correlation coefficient = 0.715

F(8,15) = 1.96 $0.05 < p < 0.2$

Intercept = 1.807

After heating the correlation between the combined effect of steroids and digoxin-like immunoreactivity failed to reach statistical significance (Table 6b).

5.7 Cross reactivity of steroids in digoxin radioimmunoassay

The respective 50% displacement (D_{50}) of the labelled digoxin by the steroids were determined from the dose response curves (Figures 7a-7c) and the results are tabulated in Table 7. 17-OHP and cortisol had almost similar D_{50} values of $1.2X10^{-4}$M and $15X10^{-4}$M respectively. Progesterone and androstenedione were about ten fold more potent in displacing 50% of labelled digoxin. The D_{50} of progesterone and androstenedione were $4.5X10^{-5}$ M and $8X10^{-5}$ M respectively. DHEAS had a D_{50} value of $2X10^{-3}$ M. Due to insolubility of oestriol and oestradiol, the 50% displacement of digoxin tracer could not be achieved, and therefore neither D_{50} nor percentage cross reactivity could be determined.

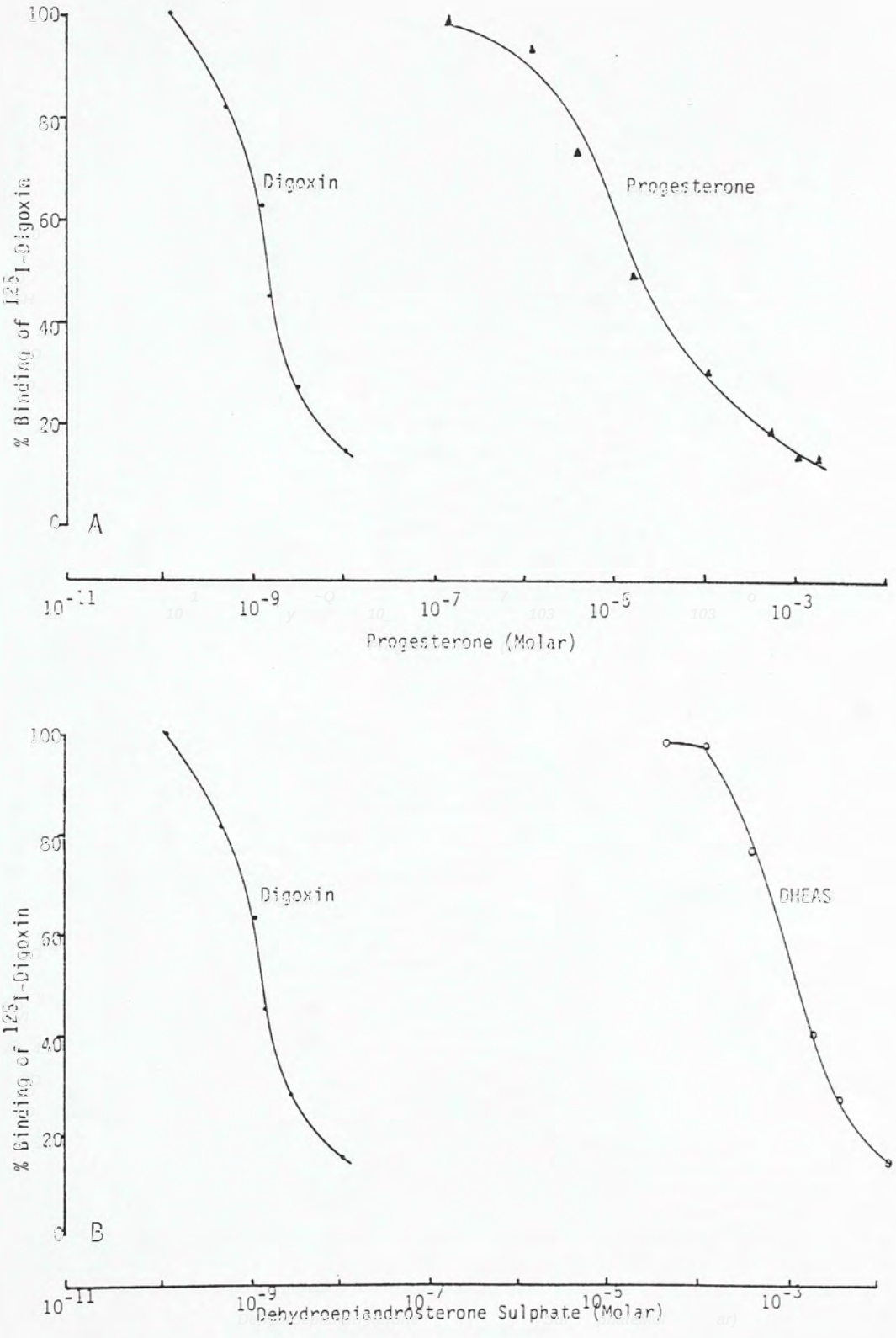


Figure 7a. Apparent displacement of [^{125}I]-digoxin from anti-digoxin antibody (RIANEN) by progesterone (panel A) and DHEAS (panel B)

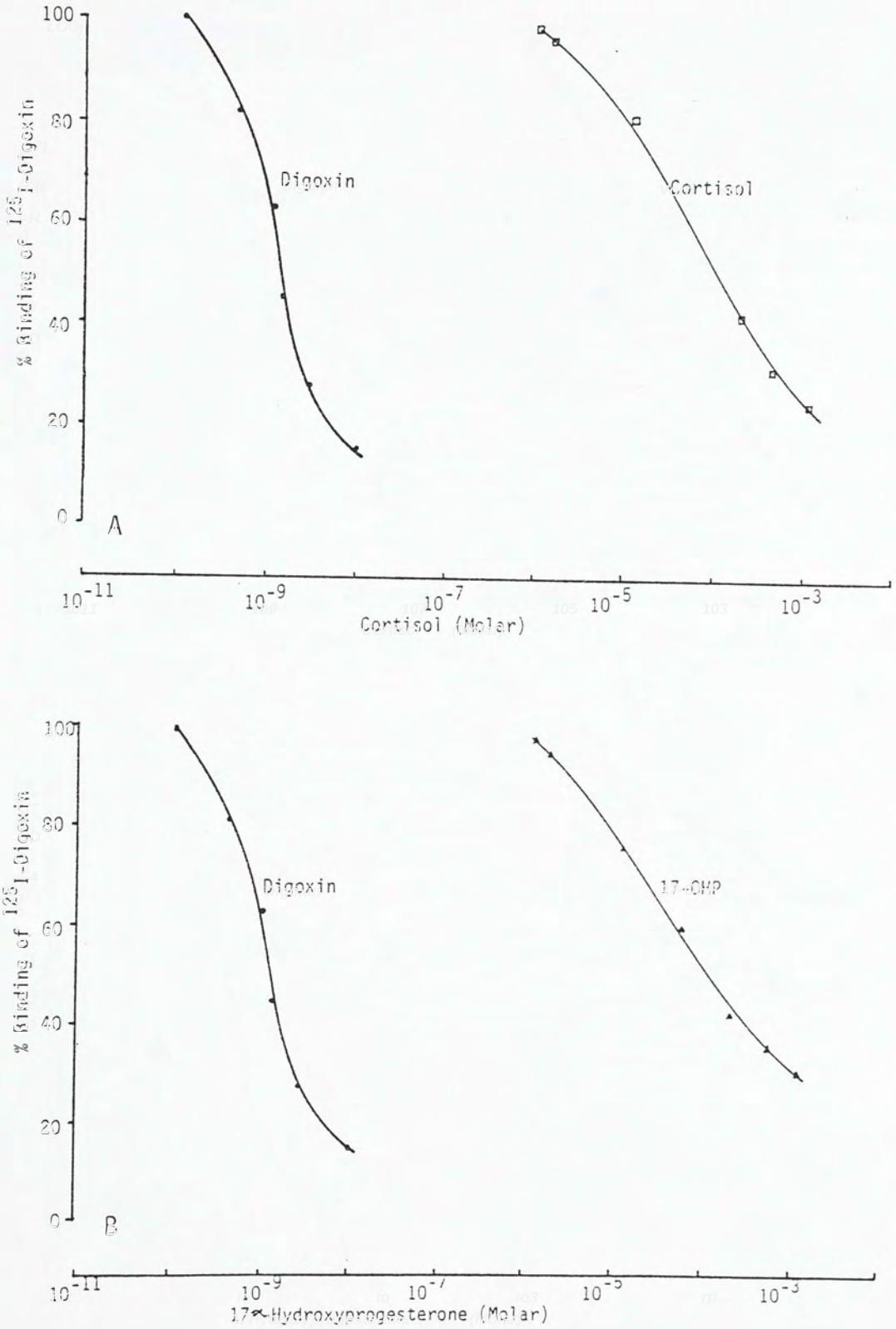


Figure 7b. Apparent displacement of [^{125}I]-digoxin from anti-digoxin antibody (RIANEN) by cortisol (panel A) and 17-OHP (panel B)

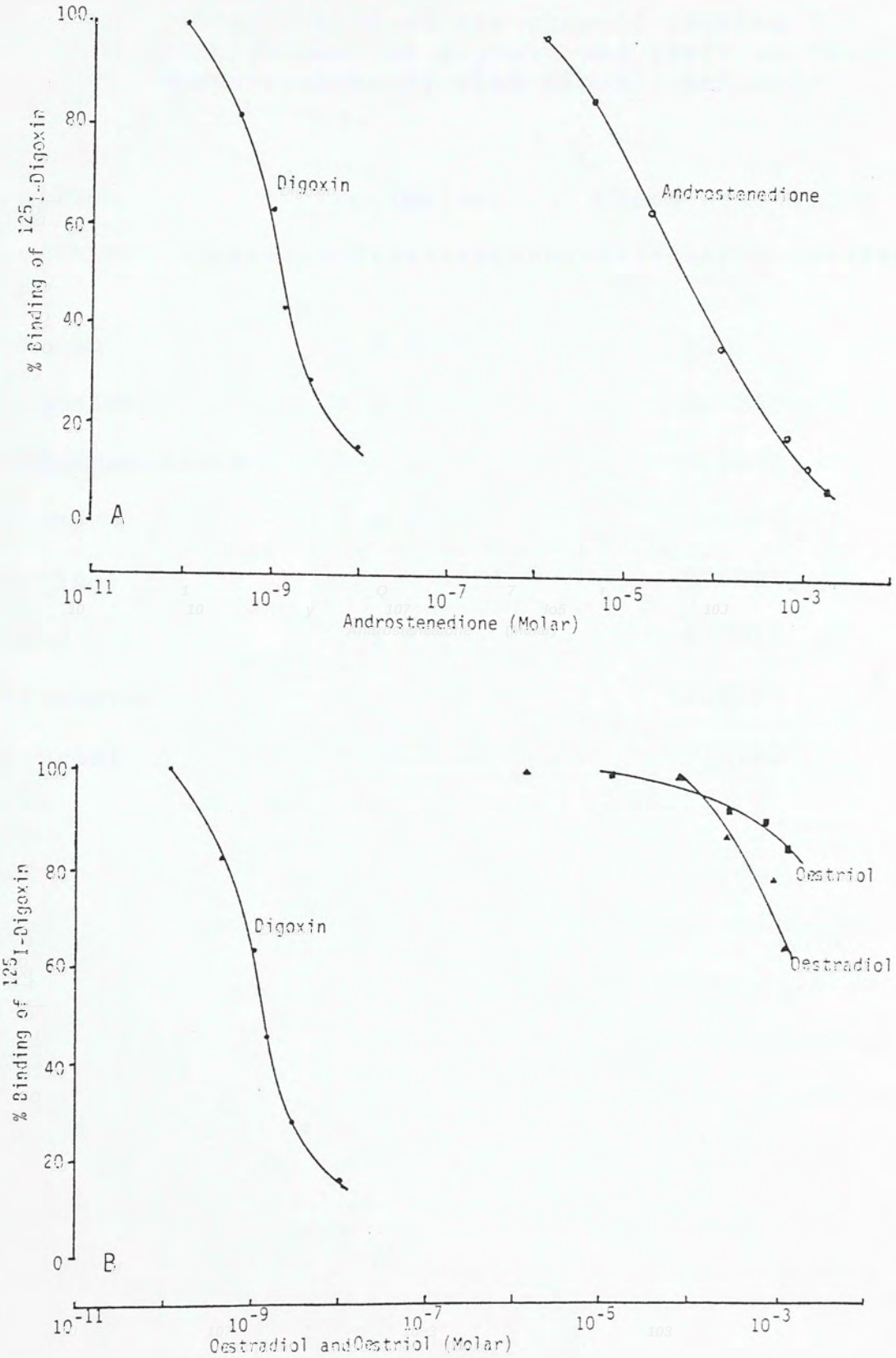


Figure 7c. Apparent displacement of [^{125}I]-digoxin from anti-digoxin antibody (RIANEN) by androstenedione (panel A), oestradiol and oestriol (panel B)

Table 7 Concentration of the steroid causing 50% displacement of digoxin and their percentage cross-reactivity with digoxin antibody

Analytes	D_{50} (molar)	Cross Reactivity (%)
Digoxin	3×10^{-9}	100
Progesterone	4.5×10^{-5}	0.0067
Androstenedione	8×10^{-5}	0.0038
17-OHP	1.2×10^{-4}	0.0025
Cortisol	1.5×10^{-4}	0.0020
DHEAS	2×10^{-3}	0.0015
Oestradiol	$>1.5 \times 10^{-3}$	<0.0002
Oestriol	$>1.5 \times 10^{-3}$	<0.0002

5.8 Inhibition of [Na^+,K^+]-ATPase by cord blood samples

The inter-assay coefficient of variation of [Na^+,K^+]-ATPase inhibition assay was found to be 12.6% (n=8).

Figure 8 gives the results of the [Na^+,K^+]-ATPase inhibition expressed as ouabain equivalent in nmol/l. The mean value was found to be 26.1 nmol/l with the range 3.4 - 48.8 nmol/l.

5.9 Correlation of digoxin-like immunoreactivity and [Na+,K+]-ATPase inhibition activity

There was no significant correlation between the DLIS level and the ATPase inhibition activity for the cord blood samples. Heat treatment of cord blood sera yielded a negative correlation between digoxin-like immunoreactivity and ATPase inhibition activity with a r value of -0.346 (Figure 9).

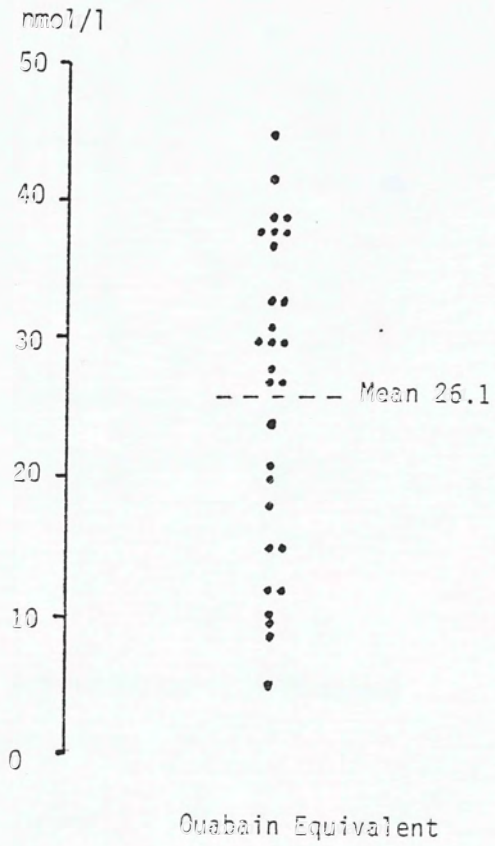
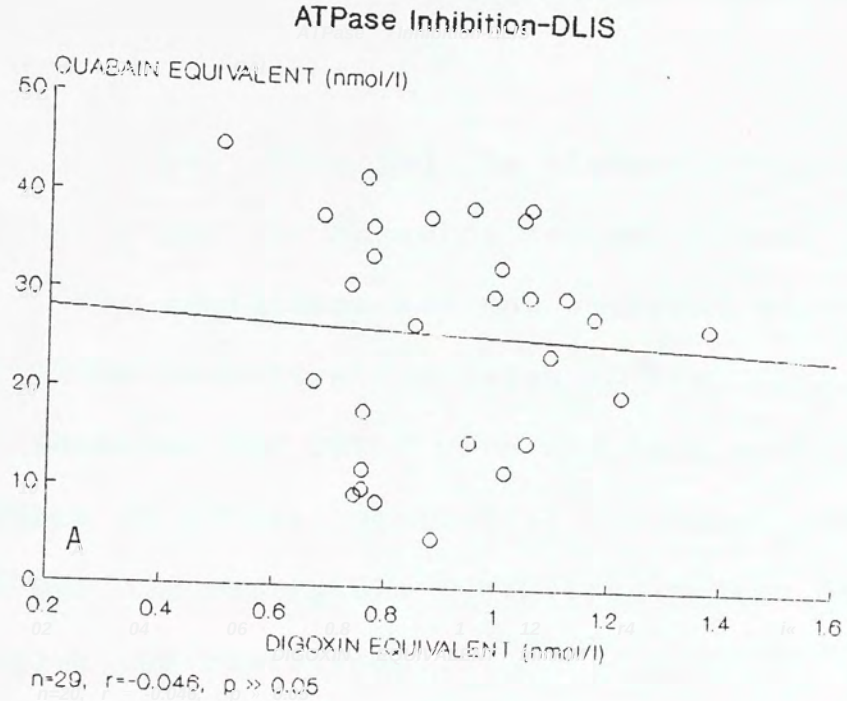


Figure 8. ATPase inhibition by the cord blood extracts expressed as ouabain equivalent (nmol/l)



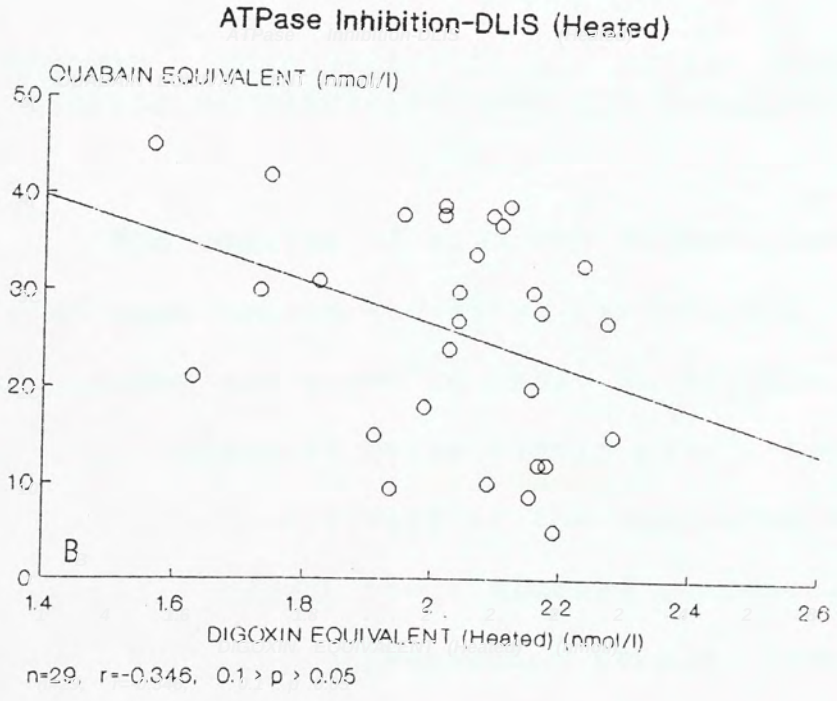


Figure 9. Correlation between ATPase inhibition and DLIS before heating (panel A) and after heating (panel B)

5.10 Apparent inhibition of [Na+,K+]-ATPase by the steroids

Progesterone had the highest inhibition of the ATPase among the steroids tested (Figure 10). However, the inhibition was not apparent at progesterone concentration below 10^{-5} M. Androstenedione and DHEAS were the next most potent inhibitors of ATPase. Oestradiol, cortisol, Oestriol and 17-OHP had negligible inhibitory action even at millimolar concentration.

5.11 Digoxin immunoreactivity and ATPase inhibitory activity of simulated cord blood samples

The results of apparent digoxin level of the simulated sera before and after Sep-pak C_{18} chromatography are shown in Table 8. Digoxin level of the *in vitro* prepared serum sample gave a low digoxin level. Inhibitory activity of the simulated sera were not detected although their steroid concentrations were comparable to each corresponding sample. Sample A, B, C and D, which covered the full range of steroids in the cord blood samples gave a low measurable level of DLIS and the ATPase inhibitory activities were insignificant.

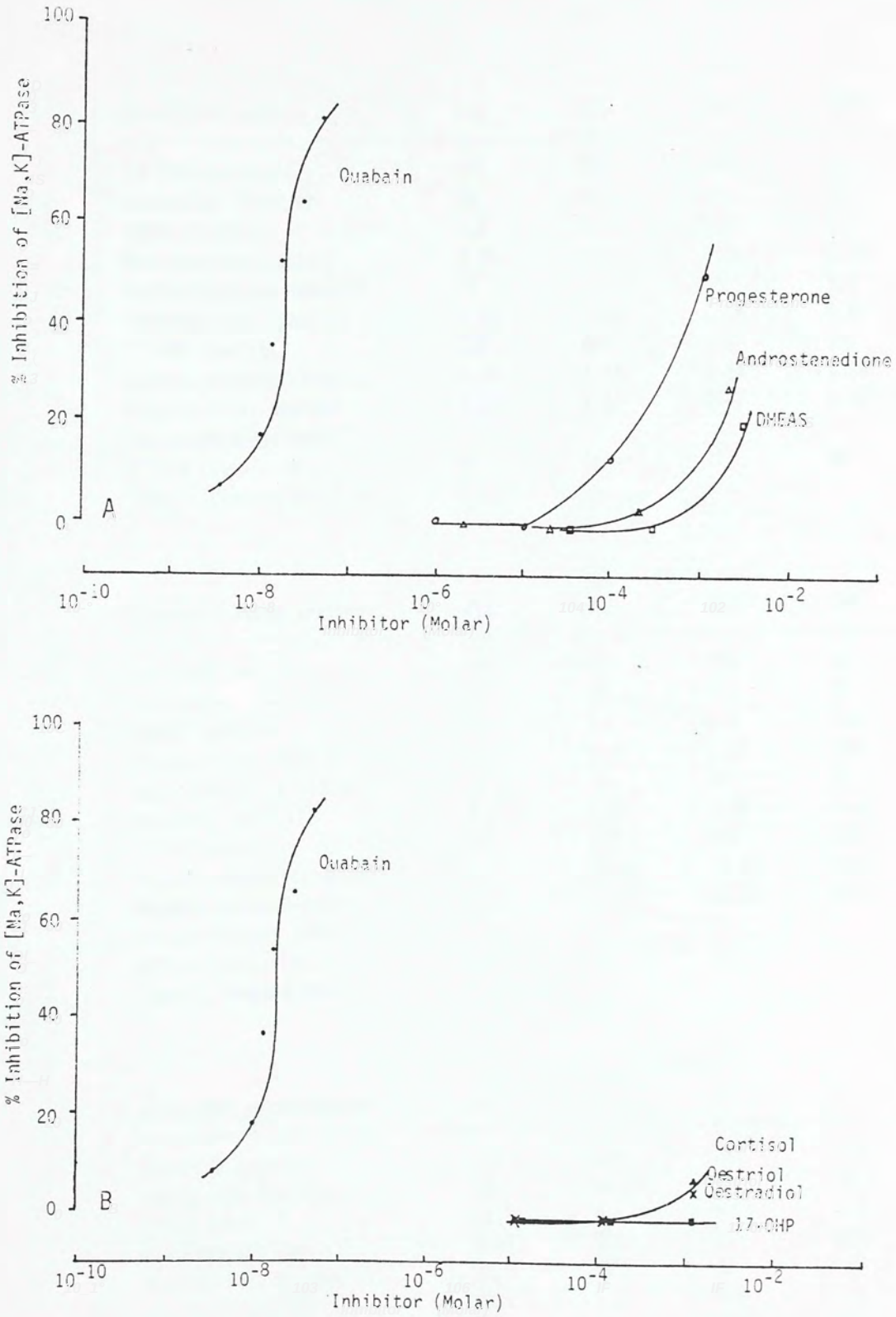


Figure 10. Apparent inhibition of $[Na^+,K^+]$-ATPase by progesterone, androstenedione, DHEAS (panel A), cortisol, oestradiol, oestriol and 17-OHP (panel B)

Table 8 Apparent digoxin and steroid concentrations in four cord blood samples, the effect of in vitro simulation of the respective cord blood serum samples

Cord blood samples	C14	C31	C43	C48
Cortisol (nmol/l)	192	226	390	94
Oestradiol (nmol/l)	28	76	116	25
DHEAS (µmol/l)	8.9	7.8	7.7	5.4
Progesterone (µmol/l)	0.84	2.27	1.56	2.14
Androstenedione (nmol/l)	12	23	25	9.9
Free Oestriol (µmol/l)	0.52	1.82	1.13	0.60
17-OHP (nmol/l)	125	402	331	148
Digoxin measured(nmol/l)	0.39	0.74	0.58	0.85
Digoxin after Sep-pak chromatography (nmol/l)	0.45	0.62	0.58	0.68
ATPase Inhibition (nmol/l Ouabain Eqv.)	45	15	27	24

Simulated serum samples	S14	S31	S43	S48
Cortisol (nmol/l)	197	197	394	99
Oestradiol (nmol/l)	26	75	131	26
DHEAS (µmol/l)	9.1	6.1	6.1	4.6
Progesterone (µmol/l)	0.91	2.29	1.53	2.28
Androstenedione (nmol/l)	13.3	20	20	10
Oestriol (µmol/l)	0.49	2.47	1.24	0.61
17-OHP (nmol/l)	107	429	429	143
Digoxin measured(nmol/l)	<0.13	<0.13	<0.13	<0.13
Digoxin after Sep-pak chromatography (nmol/l)	0.18	<0.13	<0.13	<0.13
ATPase Inhibition (nmol/l Ouabain Eqv.)	<5	<5	<5	<5

Simulated serum samples	A	B	C	D
Cortisol (nmol/l)	493	247	343	172
Oestradiol (nmol/l)	88	43	53	26
DHEAS (µmol/l)	13.1	6.6	9.1	4.6
Progesterone (µmol/l)	2.27	1.14	1.5	0.75
Androstenedione (nmol/l)	20	10	13	6.7
Oestriol (µmol/l)	12.4	6.2	8.8	4.4
17-OHP (nmol/l)	430	220	240	120
Digoxin measured(nmol/l)	0.31	<0.13	0.21	<0.13
Digoxin after Sep-pak chromatography (nmol/l)	0.36	0.17	0.22	<0.13
ATPase Inhibition (nmol/l Ouabain Eqv.)	<5	<5	<5	<5

6. DISCUSSION

Using different commercial immunoassay kits, falsely high digoxin level have been reported in neonates [30], pregnant women [17], patients with renal failure [9] and hypertension [18]. This may be attributed to the variations in antibody specificity [31] or the non-specific matrix effect [32].

Different reports have shown that the assay conditions such as the incubation time, antibody concentration and the amount of tracer can also affect the cross reactivity of digoxin immunoassay kit. By modification of the standard assay procedure for digoxin, the relative cross reactivity can be altered. It was reported that a sequential RIA analysis could reduce the cross reactivity by 20-60% relative to the standard equilibrium RIA procedure in NML kit (Nuclear Medical Laboratory, Irvine, USA). It was lowered even further by reducing the antiserum and tracer concentration [31]. In another assay, prolonging the incubation time and increasing the incubation temperature after addition of serum, labelled tracer and antiserum also reduced the interference [33].

It was demonstrated by Ho and Swaminathan that the digoxin assay could be made more sensitive for DLIS by varying the sample volume, tracer volume and antibody concentration. In doing so the sensitivity for digoxin was found to be reduced [34]. In this study, the recommended procedure of RIANEN kit was followed because it was demonstrated to have a higher detection sensitivity for DLIS.

High concentration of digoxin-like immunoreactivity was found in cord blood samples of newborn babies who had neither received any digoxin, nor had their mothers been on digoxin therapy. An average digoxin value was found to be 0.91 nmol/l with the range of 0.50-1.37 nmol/l in cord blood. These findings confirm the observations of Valdes & Graves [7].

The treatment of serum samples before the assay can also profoundly affect the results of analysis. The heat treated (5 min at 100° C) serum samples yielded a higher level of digoxin-like immunoreactivity with a mean value of 2.02 nmol/l. The ratio between heated and unheated values varied from 1.6 to 3.1 with an average of 2.3. The increase in digoxin-like immunoreactivity after heat treatment can

be due to the release of more interfering substances. These interfering substances may be non-specific substances that either sequester labelled digoxin to yield a falsely high digoxin level or bind to the digoxin antibody.

The finding of a higher level of digoxin-like immunoreactivity after heat treatment can also be due to the release of a specific DLIS. It is consistent with the hypothesis that DLIS is protein bound though not covalently. The bound DLIS are not readily released until the protein is denatured or after extensive dialysis [28]. In the study of heat treatment of oestradiol and cortisol, the assay value of these steroids did not change after heating because in these assays (direct assay), protein bound steroids are completely displaced by agents such as 8-anilino sulphonic acid. This suggests that DLIS is different from substances like oestradiol and cortisol in its properties of protein binding. At least the proportion of bound and free fraction of DLIS are certainly different from other known steroids.

In view of the steroidal nature of digoxin, it is tempting to attribute part of the the digoxin-like immunoreactivity in cord blood sample to high

level of steroid hormones and intermediates [10].

Correlation study showed that there was significant correlation between progesterone, 17-OHP, oestradiol and digoxin-like immunoreactivity in cord blood. Significant correlation was also found between the DLIS in heat treated cord sera and progesterone, 17-OHP, androstenedione and free oestriol. There was no correlation between the DLIS and cortisol, DHEAS or total oestriol in cord blood.

DHEAS [35], progesterone and its derivatives [36] have been reported as possible candidates for DLIS in the plasma of healthy adults. DHEAS was demonstrated to account for 62-100% of the total DLIS in plasma of the eleven healthy adults by Vasdev et al [35]. However, the present study showed that there was no significant correlation between concentration of DHEAS and digoxin-like immunoreactivity in cord blood. Using the same RIANEN digoxin kit as we used, the concentration of DLIS in healthy adults was found to be 36-53 pmol/l by Vasdev et al [35]. The concentration of DLIS in normal adults was far below the detection limit (0.13 nmol/l) of RIANEN digoxin kit in this study. There was no mention how the low detection limit was achieved. Unlike the situation in normal adults, the

contribution of DHEAS to digoxin-like immunoreactivity in cord blood is found to be insignificant. This is consistent with the finding of Lau et al [12].

From the multiple correlation study, the combined effect of progesterone, 17-OHP, DHEAS, androstenedione, cortisol, oertradiol and oestriol did contribute significantly to the observed level of DLIS (R=0.826, n=24). It might be due to matrix effect of the steroids in serum sample. The mechanism of the combined effect of the steroids is not known. These seven steroids may not per se account for DLIS activity in the cord blood, complicated mutual conformational or steric effect on antibody binding is possible.

The steroids included in this study did react with anti-digoxin antibody and displaced the labelled digoxin as a function of steroid concentration. Progesterone was the most potent displacer of [^{125}I]-digoxin, a concentration of 4.5X10^{-5}M caused 50% displacement of digoxin. It cross-reacted at >5X10^{-6}M. This is quite similar to the finding of Vinge et al [37]. Progesterone had the highest cross reactivity among the steroids (0.0067%). It was two times more potent than androstenedione (0.0038%), and three times more potent than 17-OHP (0.0025%) and cortisol

(0.002%). DHEAS, oestriol and oestradiol had a negligible cross reactivity at normal physiological levels in adults. The finding of low cross-reactivities for the steroids is consistent with the reports of others [8,38]. The concentration of steroid hormones required to produce a detectable DLIS level were much greater than the normal concentration found in normal adult plasma or cord serum.

In the study of biological activity of the steroids as inhibitor of ATPase, progesterone was found to have the highest inhibitory activity. It inhibited ATPase activity at concentrations of $>10^{-5}$M which is ten times higher than the concentration in the cord blood and is much above the physiological concentration in normal adults.

Androstenedione and DHEAS inhibited ATPase at concentration $>10^{-4}$M. Cortisol, oestriol, oestradiol and 17-OHP had negligible ATPase inhibitory activity at 10^{-3}M concentration.

There appeared to be a parallel relationship between the cross-reactivity and the ATPase inhibition for progesterone, androstenedione and DHEAS. Progesterone was the most potent steroid in this

regard, followed by androstenedione and DHEAS. Progesterone accounted for a mean concentration of 0.17 nmol/l of apparent digoxin level in cord blood measured by RIANEN kit. An average of 8.27% of the observed digoxin-like immunoreactivity in cord blood could be attributed to the presence of progesterone. Progesterone had also been shown to have the strongest effect on the [^{86}Rb] uptake among the anaesthetic progesterone derivatives. It was demonstrated to decrease [^{86}Rb] by 18% at millimolar concentration [37].

Reported upper limit of progesterone in the normal male adult was about 1 nmol/l and it can reach 650 nmol/l in the third trimester of pregnancy in female [29]. These concentrations in normal adult are 100 times lower than 10^{-7} M required for the cross-reactivity to occur and 10,000 times lower than 10^{-5} M needed to inhibit the ATPase activity. At normal physiological concentration of steroids it is unlikely that they contribute in a significant way to cross-react with anti-digoxin antibodies and inhibit ATPase avtivity *in vivo*.

The serum samples prepared *in vitro* to contain steroids at concentrations similar to that in

cord blood failed to give a detectable level of digoxin-like immunoreactivity by RIANEN. It may be argued that there are numerous other steroid intermediates and metabolites in cord blood, that were not assayed in the present study, but nevertheless may contribute to digoxin-like immunoreactivity. Although the major steroid intermediates in neonates eg. 3β-hydroxy-5-ene steroids, pregnenolone and pregnanetriol [38] were not included in the simulated serum, their contribution to DLIS is considered insignificant. Pregnanetriol and pregnenolone were demonstrated to have very low cross-reactivities in RIANEN digoxin kit [39].

There is lack of correlation between DLIS level and the ATPase inhibitory activity in cord blood. Although 85% recovery of DLIS immunoreactivity after Sep-Pak extraction was reported [40], partial purification of serum by Sep-Pak cartridges appeared not to be totally satisfactory for the purification of DLIS for the ATPase inhibitory test. There are over hundred compounds known to inhibit ATPase activity [41]. Some low polarity ATPase inhibitors may remain in the extract even after Sep-Pak separation. These may include lipid and some unsaturated fatty acids [9,10,11]. However, the interfering substances have yet to be identified.

The lack of specificity of present immunoassays for digoxin is a serious shortcoming for purpose of therapeutic drug monitoring. It would be useful to raise more specific antibodies to the carbohydrate component of the digoxin molecule by linking the steroid ring to the albumin and exposing the digitose sugar as the antigenic determinant [8].

7. CONCLUSION

High levels of DLIS were found in cord blood samples. Heat treatment of cord blood sample yielded even higher levels of DLIS. This is consistent with the hypothesis that there are free and bound fractions of DLIS in serum. The free to bound ratio was found to be 1:1.3 in cord blood.

Cord blood is rich in steroid hormones. Progesterone, 17-OHP and oestradiol were found to be particularly high in cord blood. Progesterone, 17-OHP and oestradiol had significant correlation with the digoxin-like immunoreactivity in cord blood. No correlations were found between DLIS and cortisol, DHEAS, total oestriol in cord blood.

Although the steroids studied did not totally account for plasma digoxin-like immunoreactivity in cord blood, the combined effect of the steroids contributed significantly to the digoxin-like immunoreactivity. 68% of the observed variation in DLIS in cord blood could be explained by the combined effect of the steroids.

Among the steroids tested progesterone had the highest cross-reactivity with digoxin antibody and was the most potent ATPase inhibitor. Progesterone concentration in the cord blood samples could only account for 2-14% of the total digoxin like immunoreactivity. It inhibited ATPase at concentration $>10^{-5}$M which is 10 times above the concentration in cord blood and 1,000 times above the concentration in normal adult.

Simulated serum cocktails containing steroids at concentrations similar to cord blood did not give a detectable level of digoxin-like immunoreactivity and ATPase inhibition. At physiological concentration seen in normal adults and cord serum, progesterone will have negligible effect on digoxin radioimmunoassay or ATPase inhibitory activity. Preliminary evidence suggests that the elevation of the major steroids only partly accounts for the level of DLIS in cord blood.

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Appendix I Steroid and DLIS levels of individual cord blood samples

Samples	Cortisol nmol/L	Oestradiol nmol/L	DHEAS umol/L	Prog umol/L	Androst nmol/L	17-OHP umol/L	Free E3 umol/L	Total E3 umol/L	DLIS nmol/L	DLIS (Heated) nmol/L	Ouabain Equivalent nmol/L
C14	192	28.2	8.88	0.84	12	0.125	0.519	8.24	0.50	1.56	45
C15	393	92.9	9.6	1.44		0.237	0.544	7.74	0.76	1.74	42
C16	261	69.1	12.42	2.54		0.34	0.904	10.12	1.00	2.23	33
C17	323	43.4	13.27	0.95		0.194	0.557	11.15	0.76	1.93	18
C19	274	25.9	6.64	1.58	13.8	0.188	0.455	5.32	0.77	2.10	37
C20	260	37.5	9.92	1.08		0.204	0.604	7.51	0.67	1.63	21
C21	326	69	8.88	2.19		0.249	1.003	7.58	1.01	2.18	12
C28	257	25.8	8	0.9	12.6	0.163	0.618	7.88	0.68	1.95	38
C29	218	37.1	5.54	1.86	13.6	0.257	0.488	7.35	0.95	2.01	39
C30	273	29.1	10.22	1.15	11	0.178	0.72	8.6	0.73	1.82	31
C31	226	76	7.8	2.27	23	0.402	1.823	12.05	0.95	2.28	15
C32	170	45.6	8.76	0.82	13.8	0.2	0.631	9.29	0.78	2.15	8.7
C33	267	41	7.23	1.4	15	0.297	0.553	6.43	0.76	2.16	12
C34	228	29.5	5.8	1.09	11.7	0.143	0.597	6.47	0.74	1.93	9.4
C35	369	59.2	6.56	1.57	14.4	0.165	0.681	9.32	0.88	2.19	5
C36	183	47.6	8.7	1.25	18.3	0.219	1.974	8.63	0.75	2.09	10
C37	357	52.6	5.3	1.73	13.6	0.179	0.968	8.54	0.99	2.04	30
C38	372	55.5	7.22	1.01	14.6	0.235	1.178	10.14	1.05	1.91	15
C39	354	50.4	11.31	1.52	15.8	0.297	0.844	9.29	1.05	2.15	30
C40	574	72.3	6.42	1.75	12.8	0.229	0.864	8.72	1.04	2.09	38
C41	162	41	7.64	1.81	12.8	0.281	1.029	9.16	0.87	2.01	38
C42	165	34.4	5.99	1.55	10.8	0.179	0.707	6.61	0.77	2.05	34
C43	390	116.1	7.73	1.56	24.9	0.331	1.131	13.19	1.37	2.27	27
C44	140	45.8	7.46	1.84	13.2	0.308	0.643	6.58	1.22	2.15	20
C45	319	35.2	2.79	1.73	8.1	0.116	0.779	9.24	1.16	2.16	28
C46	475	53.3	9.51	1.12	11.9	0.214	0.561	7.03	1.11	1.73	30
C47	172	30.6	10.9	1	11.3	0.241	0.478	8.63	0.84	2.04	27
C48	94	25	5.38	2.14	9.9	0.148	0.596	4.72	1.09	2.02	24
C49	191	70	12.44	1.61	15.3	0.327	0.842	10.89	1.05	2.11	39
MEAN	275.34	49.62	8.22	1.49	13.93	0.23	0.81	8.52	0.91	2.02	26.07
S.D.	105.56	21.31	2.39	0.45	3.66	0.07	0.35	1.83	0.19	0.18	11.35
MEAN+2S	486.47	92.25	13.00	2.39	21.25	0.37	1.51	12.27	1.29	2.38	48.77
MEAN-2S	64.22	7.00	3.44	0.60	6.60	0.09	0.11	4.77	0.53	1.67	3.38
N	29	29	29	29	24	29	29	29	29	29	29

Appendix II Apparent displacement of [125I]-digoxin from anti-digoxin antibody (RIANEN) by steroid hormones

DHEAS

Concentration	%B of 125I-digoxin
12.8 mM	16
6.4 mM	26.5
3.2 mM	41
640 uM	78.1
128 uM	99.3
64 uM	100

Progesterone

Concentration	%B of 125I-digoxin
3.18 mM	13.9
1.59 mM	14.1
795 uM	19.2
159 uM	31
31.8 uM	49.7
6.36 uM	74.4
1.27 uM	95.4
254 nM	100
127 nM	100

Cortisol

Concentration	%B of 125I-digoxin
1.38 mM	24.9
689.7 uM	31.8
344.8 uM	43.3
69 uM	62.3
13.8 uM	82
2.76 uM	97.6
1.38 uM	100
690 nM	100

Oestriol

Concentration	%B of 125I-digoxin
1.73 mM	85.6
866.9 uM	91.2
433.5 uM	93.1
86.7 uM	100
17.3 uM	100

Oestradiol

Concentration	%B of 125I-digoxin
1.84 mM	64.1
917.8 uM	78
458.9 uM	87.1
91.7 uM	98.8
18.4 uM	100

17-OHP

Concentration	%B of 125I-digoxin
1.51 mM	36.7
756.7 uM	36.9
378.4 uM	44.1
75.7 uM	62.4
15.1 uM	78.7
3.0 uM	97.1
1.5 uM	100
750 nM	100

Androstenedione

Concentration	%B of 125I-digoxin
3.41 mM	9.1
1.70 mM	13.3
852 uM	19.2
170 uM	37.2
34 uM	64.7
6.8 uM	87
1.4 uM	100
280 nM	100